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14. ABSTRACT

The aims of this proposal were to isolate and characterize prostatic epithelial stem cellsand also to determine if bone marrow (BM) cells can differentiate into prostatic tissue. Weshow that the murine prostate contains a small population of cells (<1%) capable of effluxingthe Hoechst dye by an active process (the side population, SP), indicating the presence ofcells with features of stem cells. As SP cells expressed Sca-1, a protein expressed by stemcells of other origins, we isolated Sca-1 expressing cells and determined their proliferativeability in an in vivo prostate reconstitution assay. We show that prostate stem cells residein the proximal region of ducts within the population that expresses high levels of Sca-1. Wealso show that BM stem cells can differentiate into prostatic epithelial and stromal cells.Low numbers of basal epithelial cells (~1%) are of BM origin but significant numbers ofstromal cells (~ 15%) are derived from BM. These findings may have considerable implicationsfor prostatic diseases such as cancer as gastric cancer has been shown to originate in BM-derived cells.

15. SUBJECT TERMS
Prostate stem cells, fluorescent activated cell sorting, Sca-1 antigen, bone marrow stemcells

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Introduction

This is a final report that covers the period 01/21/2004-01/20/2007 and will therefore be a summary of the purpose and results of the research completed during this time.

The aims of this proposal were to isolate and characterize prostatic epithelial stem cells and to determine if bone marrow cells could differentiate into prostatic tissues. The characterization of prostatic stem cells is important as stem cell and tumor cell biology may be closely linked and stem cells may have a role in the etiology of cancer (1-4). Stem cells and tumor cells have many common features including self-renewal, multi-drug resistance, telomerase expression and, in the instance of the prostate, androgen independence. Stem cells from a variety of tissues have been shown to actively efflux the Hoechst dye (5) through the activity of multi-drug-resistance (MDR)-like proteins (6) and this property was used to characterize and isolate them as a side population (SP).

There is also a compelling body of evidence that indicates that adult bone marrow (BM)-derived stem cells are able to differentiate into numerous types of tissues (7). Recent evidence also indicates that gastric cancer can originate from BM-derived cells (8). This data indicates that BM gives rise not only to normal differentiated cells in a variety of organs but that BM cells are also capable of being transformed within an organ and form tumors.

The aims of this proposal were therefore to determine if cells with properties expressed by side population cells were located in the prostatic tissue of mice, to examine these cells for the expression of marker proteins and to determine if they have growth properties of stem cells (Aims 1 and 2). In addition, we determined if BM cells are able to 'home' to prostate and differentiate into tissues of the urogenital tract (Aim 3).

Body

Tasks 1 and 2. To identify and isolate the side population (SP) of Hoechst-effluxing cells from the murine prostate and to identify the proteins expressed in SP cells.

A considerable amount of time and effort was expended in determining the correct conditions for obtaining and isolating a viable SP from mouse prostate. SP cells are able to efflux the Hoechst dye via a MDR-like transporter protein which is an active biological process. Verapamil (50 μM) was added to some tubes to verify the presence of a SP capable of pumping out the Hoechst dye as it inhibits the activity of MDR-like proteins. We found that verapamil addition prevented the appearance of SP cells indicating that this was an active process. The Hoechst concentration, staining time and temperature were critical for obtaining cells with SP properties that are viable. Different tissues require different conditions of labeling and the time of incubation with the Hoechst dye was varied to determination the best conditions for identifying and isolating the SP. As the Hoechst dye is toxic to cells and as viable cells are required for our in vitro studies we determined the conditions that gave us the best SP with the least toxicity.

We determined that the optimum conditions for revealing a viable SP in prostate cell digests resulted from incubating cells in Hoechst 33342 (5 μg/ml) for 90 minutes at 37•C. SP cells were evident in the proximal and in the remaining regions of prostatic ducts. We also examined the SP for evidence of two proteins known to be expressed by primitive cells - α6 integrin and Sca-1.

The results of a SP experiment are shown in Fig. 1. Propidium iodide (PI, 2μg/ml) was added before FACS analysis to eliminate dead cells from the study. After Hoechst incubation, 40% of the cells were viable. Fig.1A shows the side scatter (SSC) and forward scatter (FCS) properties (i.e., the size and granularity of the cells) of the viable Hoechst treated cell population. Fig. 1B shows the Hoechst excluding SP (R2) is 0.65% of the viable population (R1) (1036 SP cells from 159,679 viable cells in R1). The expression of α6 integrin and Sca-1 was determined on these cells after incubating the Hoechst treated cells with either PE-labeled (Sca-1) or FITC-labeled (α6 integrin) antibodies to these antigens. Isoytpe matched control antibodies were added to some cells as controls (Fig. 1C). Fig. 1D indicates that 87.9% of the cells in the SP expressed both Sca-1 and α6 integrin whereas 8.1% of SP cells expressed Sca-1 only. We next examined the SSC and FSC properties of the SP to determine the sizes and granularity of these cells and found that the SP is comprised of cells that are small to medium sized with little granularity (Fig.1E).

These results show that the murine prostate contains cells capable of effluxing the Hoechst 33342 dye by an active process (the side population), indicating that cells with features of stem cells are found in the prostate gland. In addition we show that 96% of the SP cells express Sca-1, a protein shown to be expressed by stem cells of other origins (9, 10). Most of the Sca-1 expressing cells also express α6 integrin which is also found on many primitive cell types (11-13).

Task 3. To determine the growth potential of SP and non-SP cells

We next examined the growth properties of the FACS sorted SP cells by suspending them in collagen gels and determining the numbers and sizes of prostatic ducts formed after 2 weeks incubation at 37°C. We have previously found that primitive prostatic cells have an increased duct-forming efficiency and also form larger and more complex ducts (14). We found that SP cells formed 21.3 ± 3.8 ducts compared with 6.3 ± 0.6 ducts for non-SP cells ($p < 0.001$; Fig. 2A). The sizes and complexity of the ducts formed from SP cells was also greater than those of non-SP cells (Fig. 2B, C).

These data indicate that SP cells have a greater proliferative capacity than non-SP cells as would be expected if SP cells are of more primitive origin than non-SP cells.

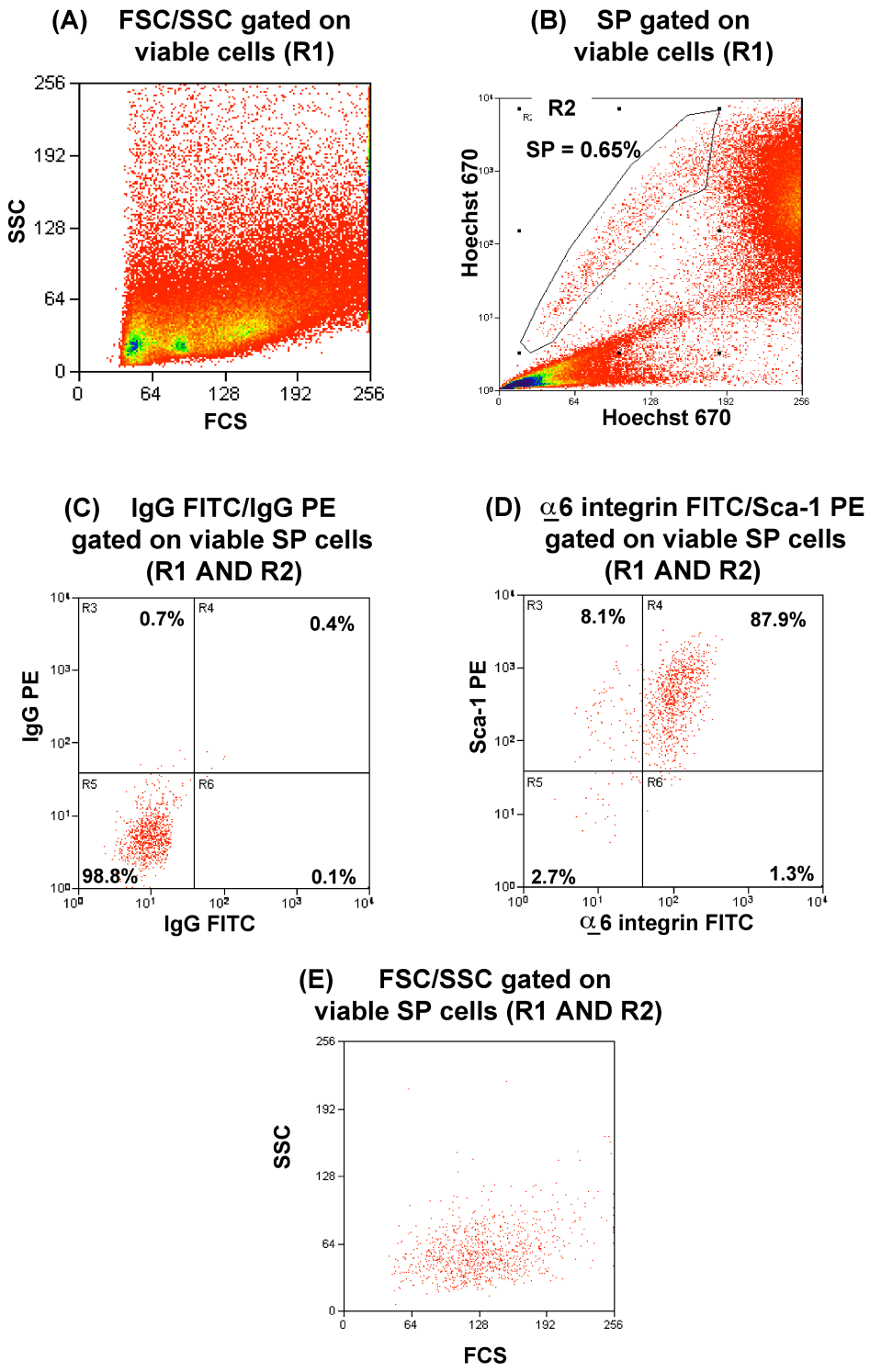

Fig. 1. Prostate contains SP cells that express Sca-1 and α6 integrin.
A. SSC and FCS properties of the viable Hoechst stained population.
B. Viable SP cells represent 0.65% of the total population. **C.** Hoechst stained cells were incubated with isotype-matched control PE and FITC labeled antibodies. **D.** SP cells express both Sca-1 and α6 integrin. **E.** SSC and FSC properties of the SP cells.

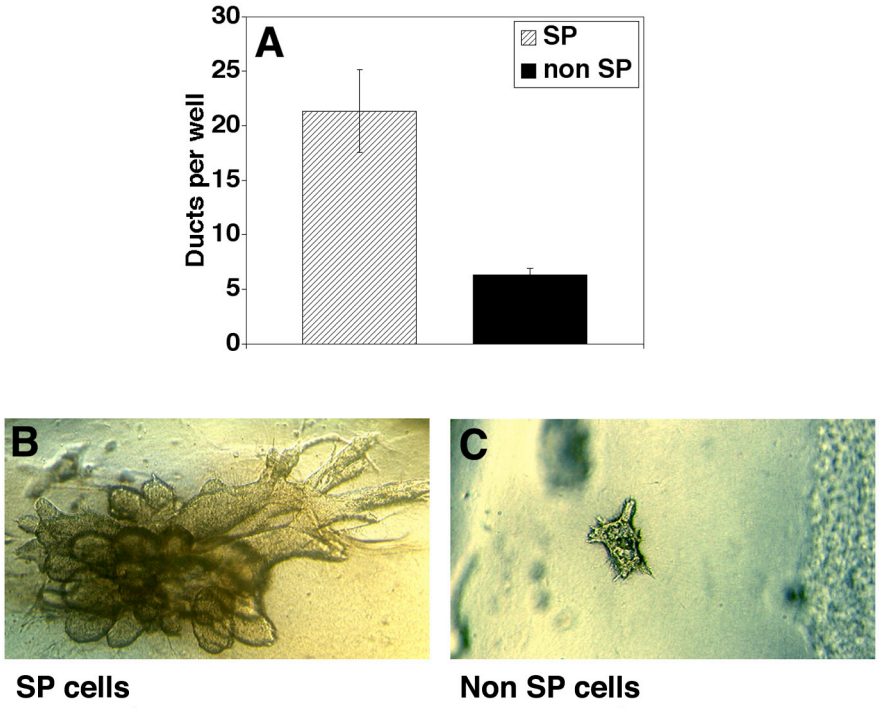

Fig 2. SP cells have greater proliferative potential than non-SP cells.
A. FACS sorted SP and non-SP cells (4000/well) were seeded in collagen gels and the numbers and sizes of ducts were determined after 14 days.
B and C. SP cells (B) formed larger and more complex structures in collagen gels than non-SP cells (C).

We were unable to do the in vivo experiments proposed in Task 3 due to the low numbers of SP cells obtained in the prostate (<1%) and due to their poor viability as they have an in vitro cloning efficiency of ~0.5%. This resulted in so few cells with the ability to proliferate that we were unable to obtain sufficient numbers of cells to insert under the renal capsule of recipient animals or to further fractionate and isolate them based on the expression of Sca-1 and α6 integrin. The Hoechst dye 33342 has been found to be toxic to some cells and can result in low viability after FACS sorting. As this was our experience we were unable to proceed with this approach. We were also unable to do the in vivo intraprostatic inoculations of GFP-tagged FACS isolated SP cells proposed in Task 4. The low numbers of SP cells with growth capacity meant that sufficient GFP-tagged SP cells could not be obtained for these intraprostatic in vivo studies.

As we had shown that almost all the SP cells expressed Sca-1 and as SP cells have been shown to have stem cell properties in other systems we proceeded to isolate the Sca-1 expressing population from the proximal and remaining regions of ducts and compare their properties in in vivo assays. We found that Sca-1 is highly expressed in the proximal region of prostatic ducts and that prostatic stem cells can be isolated based on high levels of expression of Sca-1 (15).

We found that 52 ± 11% of proximal cells express Sca-1 compared with 18 ± 7% of cells in the remaining regions of ducts. The proximal region also had far more cells (10 ± 1%) expressing high levels of Sca-1 (Sca-1^{high}) than remaining ductal regions (2 ± 1%) (15). We also found that the proximal region is considerably enriched (28 ± 4%) in Sca-1 expressing cells that co-express α6

integrin and Bcl-2 - two other antigens shown to be expressed by stem cells – than remaining regions (1 ± 1%) (15). These results show that there are striking differences in the distribution of cells expressing Sca-1, α6 integrin and Bcl-2 in different ductal regions. Cells with high levels of Sca-1 are predominantly confined to the proximal region and triple labeled cells with high levels of Sca-1 are almost exclusively confined to this region (15).

Sca-1 expressing cells have high in vivo regenerative potential

The ability to regenerate tissue in vivo is a characteristic of stem cells and this property has been used to identify various antigens, including Sca-1, as stem cell markers (10, 16). We therefore determined the growth potential of Sca-1 expressing cells isolated (using magnetic beads and antibodies to Sca-1) from the proximal and the remaining ductal regions and compared their proliferative potential in vivo with cells that did not express this antigen. These populations were combined with cells isolated from the urogenital sinus mesenchyme (UGM) of 18 day old embryos (inductive mesenchyme for prostatic tissue), inserted under the renal capsule (RC) of recipient male animals and the amount of prostatic tissue generated was measured after 8 weeks. Sca-1 expressing cells isolated from the proximal region formed significantly more prostatic tissue (17.1 fold; $p < 0.001$) than was obtained from the Sca-1 depleted proximal population (Fig. 3) (15).

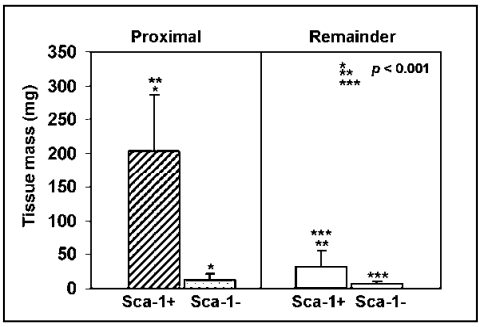

Fig. 3. Sca-1+ cells have greater in vivo regenerative capacity than Sca-1- cells. The growth of Sca-1+ and Sca-1- cells (10^5 cells) that were isolated from either the proximal or the remaining regions and transplanted under the RC was measured after 8 weeks. Sca-1+ cells, obtained from the proximal region formed 17.1 fold more prostatic tissue than Sca-1- cells ($p < 0.001$). Sca-1+ cells, obtained from the remaining ductal regions, had far less growth potential than Sca-1+ proximal cells ($p < 0.001$). Sca-1- cells from the remaining regions showed less growth than Sca-1+ cells from this region ($p < 0.001$).

Sca-1 expressing cells isolated from the remaining ductal regions also formed prostatic tissue under the RC (~32 mg) but formed far less tissue than observed for Sca-1 expressing cells isolated from the proximal region (~200 mg), indicating that these Sca-1 expressing populations differ markedly in their in vivo growth potential ($p < 0.001$). Sca-1 depleted cells isolated from the remaining regions of ducts formed very little sub-RC tissue.

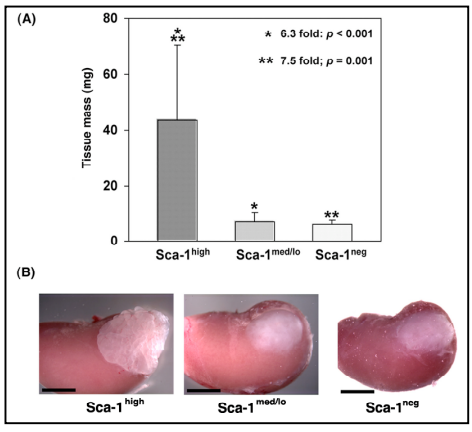

Fig. 4. Sca-1^{high} cells have greater in vivo regenerative capacity than cells that express lower levels of Sca-1. Cells were isolated from the proximal region and FACS sorted into Sca-1^{high}, Sca-1$^{med/lo}$ and Sca-1^{negative} fractions. The cell populations (3 x 10^4) were transplanted under the RC and the growth of prostatic tissue was measured after 10 weeks.
(A) Sca-1^{high} cells formed 6.3 fold more prostatic tissue than Sca-1$^{med/lo}$ cells ($p < 0.001$) and 7.5 fold more prostatic tissue than Sca-1^{negative} cells ($p = 0.001$).
(B) Prostate tissue initiated with 3 x 10^4 Sca-1^{high}, Sca-1 $^{med/lo}$ or Sca-1^{negative} cells isolated by FACS from the proximal region of ducts. Bars = 3 mm.

As our FACS data showed that cells expressing high levels of Sca-1 were confined predominantly to the proximal region of ducts, we determined whether Sca-1^{high} cells had greater growth potential than cells with medium/low Sca-1 expression. Proximal cell populations were FACS

sorted into fractions containing cells with high median fluorescence intensity (MFI) (Sca-1^{high}), medium to low MFI (Sca-1$^{med/lo}$) and no Sca-1 expression (Sca-1^{negative}) and inserted under the RC of recipient animals. Sca-1^{high} cells formed significantly more prostatic tissue than Sca-1$^{med/lo}$ or Sca-1^{negative} cells (Fig. 4) (15).

Although the tissue growth obtained was less than in experiments using magnetic beads (due to the stress that the cells undergo during FACS sorting; note also that far fewer FACS sorted cells (3 x 10^4) were used), these results show that almost all of the in vivo regenerative potential is confined to cells that express high levels of Sca-1. The prostatic tissue obtained from Sca-1^{high} cells had normal prostatic histology comprising basal and luminal cells lining prostatic ducts. The lumina of the ducts contained abundant amounts of secretory material. In contrast, the tissue arising from Sca-1$^{med/lo}$ and Sca-1^{negative} cells contained more stroma with less of an epithelial component and little secretory material was noted within the ducts.

These results show that cells expressing Sca-1 have considerably more growth potential than those lacking this antigen and that the proliferative ability within the Sca-1 expressing proximal cells resides in cells that express high levels of this antigen. They also show that Sca-1 expressing cells residing in the proximal region are more primitive than those Sca-1 expressing cells in the remaining ductal regions as they have far higher regenerative capacity. These data indicate that stem cells reside within the Sca-1 expressing population in the proximal region, whereas cells with more limited growth potential (transit-amplifying cells), reside within the Sca-1 expressing cells in the remaining ductal regions.

We have also shown that α6 integrin expressing cells are enriched in the proximal region of ducts and that they form more prostatic tissue under the RC than those depleted of this antigen (17). In addition we find that proximal stem cells withstand prolonged androgen deprivation and that primitive proximal cells can be passaged through four generations of subrenal capsule grafts indicating extensive self-renewal capability (17).

Task 5. To determine if bone marrow-derived cells can differentiate into and 'home' to organs of the urogenital tract.

The aim of these experiments was to determine if bone marrow stem cells can migrate to the prostate and differentiate into prostatic epithelial or stromal cells. There is a considerable recent literature indicating that adult-derived bone marrow stem cells maintain extensive plasticity and differentiate into multiple organs in the mouse (7, 18-20). In addition, gastric cancer has been shown to originate from bone marrow-derived cells (8) indicating that bone marrow-derived cells may also be relevant to the process of prostatic carcinogenesis.

GFP-expressing bone marrow cells were obtained from GFP-expressing C57Bl6 mice (Jackson Labs, ME) and transplanted into irradiated recipient male mice. Engraftment was ascertained by analyzing peripheral blood of recipient animals for evidence of GFP-expressing cells. The recipient animals were castrated to induce prostatic involution. After 14 days testosterone pellets (Innovative Research, FL) were implanted subcutaneously to regenerate the prostate as we expected circulating bone marrow stem cells to engraft more readily into regenerating prostate tissue. As engraftment was very low we cycled the mice by adding and removing androgen. In addition, to promote stem cell engraftment, GFP-expressing hematopoietic stem cells were mobilized from the bone marrow during androgen administration with GCSF (granulocyte colony stimulating factor; 300 μg/kg/day).

As can be seen in Table 1 low numbers of GFP-expressing BM cells engrafted into the epithelium and engraftment was increased by cycling with GCSF. The engrafted GFP+ cells were primarily basal (CK5+) and no luminal GFP+ cells were noted. The GFP+CK5+ cells represented only ~1% of total prostate epithelial cells and between 3-6% of basal cells. Approximately 70% of the engrafted GFP+ cells expressed CK5. Most of the engrafted GFP+ cells lacked the expression of CD45 indicating that they no longer expressed this hematopoietic specific marker and had differentiated into prostatic basal cells.

Table 1 Engraftment of GFP^+ bone marrow cells into prostate epithelium

	3cycles +GCSF	**7cycles +GCSF**	**7cycles no GCSF**
GFP^+/total (%)	1.2±0.4#	1.9±0.4	1.1±0.2#
GFP^+CK5^+/total (%)	0.9±0.3#	1.4±0.0	0.8±0.1*
GFP^+CK5^+/GFP^+ (%)	68.4±6.4	69.0±0.7	68.5±7.6
GFP^+CK5^+/basal (%)	3.7±1.1	6.0±2.1	3.0±0.1#
GFP^+CD45^-/GFP^+ (%)	76.2±13.4	75.0±7.6	73.1±5.4

Mice were subjected to 3-7 cycles of androgen depletion and replenishment and were treated with or without GCSF during each cycle**.** Each value represents the mean ± SD. Values are significant at # $p<0.05$ and * $p<0.01$ when comparing 7 cycles + GCSF to 3 cycles + GCSF or 7 cycles without GCSF.

In contrast to the low engraftment seen in the epithelium significant numbers of GFP+ BM cells engrafted into prostatic stroma (Table 2) and cycling with androgens in the presence of GCSF significantly increased this engraftment. After 7 cycles with GCSF 20% of the total prostatic stromal cells were GFP+ and 29% of the vimentin expressing cells expressed GFP. Approximatley 70% of the total GFP+ cells in the stroma were also vimentin+ (~ 50% of stromal cells are vimentin expressing in the prostate). More than 70% of the stromal GFP+ cells lacked CD45 indicating that they were no longer expressing hematopoietic markers and had differentiated into prostatic stromal cells.

Table 2 Engraftment of GFP$^+$ bone marrow cells into prostate stroma

	3 cycles +GCSF	7 cycles +GCSF	7 cycles no GCSF
GFP$^+$/total stroma (%)	8.3±2.2*	20.7±4.3	10.8±1.9*
GFP$^+$vimentin$^+$/total (%)	7.2±1.7*	15.3±3.6	7.5±1.8*
GFP$^+$vimentin$^+$/vimentin$^+$ (%)	25.7±8.5	29.3±5.0	29.1±1.2
GFP$^+$vimentin$^+$/GFP$^+$ (%)	70.9±10.0	73.0±6.8	73.3±11.3
Vimentin$^+$/total stroma (%)	31.4±12.8#	51.2±6.4	27.5±8.2*
GFP$^+$CD45$^-$/GFP$^+$ (%)	70.7±1.0	78.1±4.1	77.5±10.6

Mice were subjected 3-7 cycles of androgen depletion and replenishment and were treated with or without GCSF during each cycle**.** Each value represents the mean ± SD by 2-tailed t-test. Values are significant at # $p<0.05$ and * $p<0.01$ when comparing 7 cycles + GCSF to 3 cycles + GCSF or 7 cycles without GCSF.

Fig. 5 shows that BM contains cells capable of differentiating into prostatic basal epithelial cells but that this is a rare event. In contrast BM differentiates into significant numbers of prostatic stromal cells indicating that prostatic stroma derives largely from circulating BM cells. These findings may have considerable implications for the etiology of prostatic diseases as gastric cancer has been shown originate from bone marrow-derived cells (8).

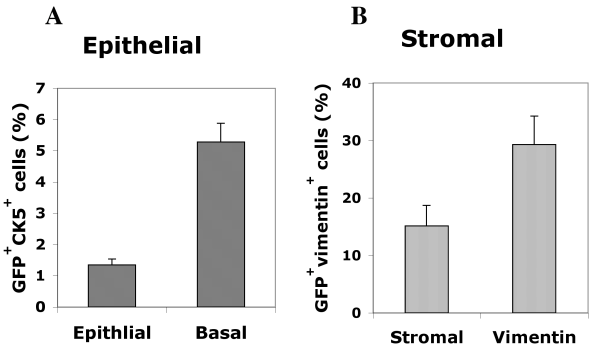

Fig. 5 Contribution of BM-derived cells to prostatic epithelial and stromal cells. Data was obtained from mice that received transplants of GFP-expressing BM cells. All mice received G-CSF mobilization and were subjected 3-7 cycles of androgen depletion and replenishment. (**A**) Frequency of GFP$^+$ BM cells in the epithelium. The percentages were determined by dividing GFP$^+$CK5$^+$ cells by total epithelial or total basal cells. **(B)** Frequency of GFP$^+$ BM cells in the stroma. The percentages were determined by dividing GFP$^+$vimentin$^+$ cells by total stromal or total vimentin$^+$ cells. It indicates that there are significantly more GFP$^+$ BM cells in the stroma than in the epithelium.

Key research accomplishments

1) Conditions for obtaining a Hoechst 33342 dye excluding population of viable prostate stem cells (a side population of prostatic stem cells) have been defined.

2) The prostate SP also expresses two antigens found on stem cells of other origins, namely Sca-1 and α6 integrin.
3) SP cells have greater proliferative potential than non-SP cells in collagen gel assays designed to measure the numbers and sizes of prostatic ducts that arise from individual cells.
4) The proximal region of murine prostatic ducts is considerably enriched in cells that express high levels of the cell surface antigen, Sca-1.
5) Sca-1-expressing cells were isolated from the proximal region and remaining regions of ducts and assayed for proliferative potential in an in vivo prostate reconstitution assay. This showed that proximal Sca-1 expressing cells had far greater growth potential than Sca-1 expressing cells isolated from remaining regions of ducts which are likely to be a post-stem cell (transit-amplifying) compartment.
6) Almost all of the proliferative activity resided in the cells expressing high levels of Sca-1 indicating that prostatic stem cells reside within the Sca-1^{high} compartment.
7) Bone marrow cells can 'home' to the prostate and are able to differentiate into both prostatic epithelium (basal cells) and stroma (vimentin expressing cells). However, significantly more stromal cells are of bone marrow origin than epithelial cells.

Reportable outcomes

1) Publications - see attached manuscripts:
P.E. Burger, X Xiong, S Coetzee, S.N. Salm, D. Moscatelli, K Goto, E. L. Wilson. Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with a high capacity to reconstitute prostatic tissue. Proc Natl Acad Sci USA 102; 7180-7185 (2005).

K Goto, SN Salm, S Coetzee, X Xiong, PE Burger, E Shapiro, H Lepor, D Moscatalli, EL Wilson. Proximal prostatic stem cells are programmed to regenerate a proximal-distal ductal axis. Stem Cells 24; 1859-1868, 2006.

2) The PI presented a talk incorporating data from this proposal at the NIH sponsored Cellular Niches Workshop, May 16-17, 2005, Bethesda, MD
The properties and regulation of prostatic stem cells and their relevance to prostatic diseases.
E Lynette Wilson, Ken Goto, Sarah Salm, Patricia Burger, Sandra Coetzee, Xiaozhong Xiong, Ellen Shapiro, Herbert Lepor and David Moscatelli.

3) The following posters incorporating data from this proposal were presented at the Cellular Niches Workshop, May 16-17, 2005.
Sca-1 expression identifies a progenitor/stem cell population in the proximal region of murine prostatic ducts with a high capacity to reconstitute prostatic tissue
Patricia E. Burger, Sandra Coetzee, Xiaozhong Xiong , Sarah N. Salm, David Moscatelli, Ken Goto and E. Lynette Wilson.

Bone Marrow Cells are able to generate prostatic epithelial and stromal cells.
Xiaozhong Xiong, Ken Goto, Yasuhiro Ebihara, Sarah Salm, Sandra Coetzee, Christopher Ontiveros, David Moscatelli, Makio Ogawa and E Lynette Wilson.

4) NIH funding is currently been applied for based on work supported by the grant.

Conclusions

We have shown that the prostate contains a small population of cells (~0.6%) that exclude the Hoechst dye and whose ability to exclude the dye is inhibited by verapamil. Almost all cells (96%) of this side population (SP) express the antigen Sca-1 and most of them (88%) co-express Sca-1 with ∝6 integrin. As the Hoechst dye was toxic to the small SP, sufficient numbers of cells could not be obtained for in vivo prostate reconstitution experiments to determine their stem cell origin. As most SP cells expressed Sca-1, we isolated cells based on Sca-1 expression and showed that the stem cell population in the prostate resides within that fraction of cells expressing high levels of this antigen. We also show that cells expressing high levels of Sca-1 express ∝6 integrin and Bcl-2, an anti-apoptotic protein found in tumor cells and stem cells.

We have therefore, for the first time, provided a profile of prostatic stem cells based on protein expression that enables their purification and assay. This population can now be compared with prostate tumor cell populations to determine their similarity or differences. As tumor stem cell populations have recently been isolated we expect that normal stem cells and tumor stem cells will have overlapping features. The identification of prostate stem cells may enable development of new therapies which may ablate both normal and tumor stem cells. As the prostate is not an essential organ the ablation of normal prostatic stem cells in conjunction with carcinoma cells will not adversely affect the health of the patient.

Our data also indicate that bone marrow stem cells can differentiate into both epithelial and stromal compartments of the prostate with significantly more prostatic stromal cells than epithelial cells being of bone marrow origin. This finding may have considerable implications for the etiology of prostatic diseases as gastric cancer has been shown originate from bone marrow-derived cells (8).

Personnel supported by this grant

E Lynette Wilson Effort 15%
Rashmi Gupta Effort 100%

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Sca-1 expression identifies stem cells in the proximal region of prostatic ducts with high capacity to reconstitute prostatic tissue

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We previously showed that prostatic stem cells are concentrated in the proximal regions of prostatic ducts. We now report that these stem cells can be purified from isolated proximal duct regions by virtue of their high expression of the cell surface protein stem cell antigen 1 (Sca-1). In an *in vivo* prostate reconstitution assay, the purified Sca-1-expressing cell population isolated from the proximal region of ducts was more effective in generating prostatic tissue than a comparable population of Sca-1-depleted cells (203.0 ± 83.1 mg vs. 11.9 ± 9.2 mg) or a population of Sca-1-expressing cells isolated from the remaining regions of ducts (transit-amplifying cells) (31.9 ± 24.1 mg). Almost all of the proliferative capacity of the proximal duct Sca-1-expressing cell population resides within the fraction of cells that express high levels of Sca-1 (top one-third), with the proximal region of prostatic ducts containing 7.2-fold more Sca-1^{high} cells than the remaining regions. More than 60% of the high-expressing cells coexpress α6 integrin and the antiapoptotic factor Bcl-2, markers that are also characteristic of stem cells of other origins. Further stratification of the phenotype of the stem cells may enable the development of rational therapies for treating prostate cancer and benign prostatic hyperplasia.

prostate | α6 integrin | Bcl-2

Stem cell biology and tumorigenesis may be closely linked, and stem cells may have a role in the etiology of cancer (1–5). Stem cells and tumor cells have many common features, including self-renewal, multidrug resistance, telomerase expression, and, in the case of the prostate, androgen independence. Prostatic stem cells do not require androgens for survival, as evidenced by normal prostatic regeneration after >30 cycles of androgen ablation and supplementation, which results in involution and normal regeneration of this gland (6). Because prostatic carcinoma usually progresses to an androgen-independent tumor (which may reflect a stem cell-like phenotype), an understanding of prostate stem cell biology is important for devising preventative or therapeutic approaches for prostate cancer. In addition to being a source of carcinomas, stem cells may also give rise to benign prostatic hyperplasia (7). The isolation and characterization of these stem cells is likely to increase our understanding of normal prostate physiology, and it may also lead to new therapeutic approaches for two of the most common diseases afflicting men.

The murine prostate consists of a branched ductal network with each duct consisting of proximal (adjacent to the urethra), intermediate, and distal regions. Actively proliferating cells (transit-amplifying cells) are located in the distal region of the ducts (8), whereas cells with stem cell features are concentrated in the proximal ductal region (9). Thus, cells in the proximal region are quiescent and have high proliferative potential, and isolated single cells from this region can give rise to branched glandular ductal structures *in vitro* (9). In addition, cell suspensions derived from the proximal region form significantly more prostatic tissue in an *in vivo* transplantation model than those obtained from other prostatic regions. Furthermore, cells obtained from this transplanted tissue are again able to give rise to prostatic tissue when reinoculated into other animals (unpublished data), confirming the presence in the proximal region of stem cells with regenerative capacity.

Because stem cells in other organs have been identified by their expression of specific antigens, such as a cell surface protein known as stem cell antigen 1 (Sca-1), α6 integrin, and Bcl-2, we determined whether these antigens could be used to identify the stem cell population in the proximal region of ducts. Sca-1 is expressed by stem/progenitor cells from a variety of tissues, such as hematopoietic tissue (10), cardiac tissue (11), mammary gland (12), skin (13), muscle (14), and testis (15). α6 integrin (CD49f) is expressed by primitive cells in the liver (16) and skin (17), and anti-α6 integrin antibodies have been used to enrich for spermatogonial stem cells from mouse testis (18). Bcl-2, an antiapoptotic protein (19), may protect primitive cells from death and is expressed by hematopoietic, keratinocyte, and colon stem cells (20–22).

We have identified a candidate population of prostatic stem cells in the proximal region of murine prostatic ducts that expresses high levels of Sca-1, in conjunction with α6 integrin and Bcl-2. Cells with these properties are almost absent from the remaining regions of ducts. We show that Sca-1-expressing cells isolated from the proximal region regenerate abundant normal functional prostatic ducts in an *in vivo* transplantation assay, whereas cells that do not express this antigen form very little tissue. These results establish that prostatic stem cells reside within the Sca-1-expressing population in the proximal region of ducts and provide a means of isolating the stem cells for further characterization.

Materials and Methods

Animals. C57BL/6 mice, athymic nude mice, and CDIGS rats were housed in the animal research facilities of the University of Cape Town or New York University, and all experiments were performed in compliance with institutional review board requirements.

Antibodies and Control Immunoglobulins (IgGs). Fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Sca-1, rat anti-mouse

Abbreviations: MFI, mean fluorescence intensity; PE, phycoerythrin; Sca-1, stem cell antigen 1; UGM, urogenital sinus mesenchyme.

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Table 1. Expression of Sca-1, α6 integrin, and Bcl-2 by cells from the proximal region of prostatic ducts compared with cells from the remaining ductal regions

Phenotype	No. of experiments	Proximal expression, %	Remainder expression, %	Increase, fold	*P*
Sca-1$^+$	16	51.8 ± 10.5	17.7 ± 7.2	2.9	<0.0001
α6 integrin$^+$	13	40.8 ± 10.0	21.1 ± 11.4	1.9	<0.0001
Bcl-2$^+$	12	42.1 ± 7.0	27.5 ± 8.2	1.5	<0.0001
Sca-1$^+$ α6 integrin$^+$ Bcl-2$^+$	3	27.5 ± 4.4	1.4 ± 0.8	19.6	<0.01
Sca-1^{high}*	12	15.9 ± 5.2	2.2 ± 1.4	7.2	<0.00001
Sca-1^{high}* α6 integrin$^+$ Bcl-2$^+$	3	9.8 ± 1.2	0.1 ± 0.06	98.0	<0.01

*Sca-1+ cells with fluorescence intensities in the upper one-third were defined as Sca-1^{high} cells.

α6 integrin (CD49f) FITC, and rat IgG 2a FITC were obtained from BD Biosciences, Bedford, MA. Phycoerythrin (PE)-conjugated rat anti-mouse Sca-1, rat IgG-2a PE, rat IgG, mouse anti-mouse CD16/32, rat anti-mouse Sca-1 biotin, rat IgG2a biotin, and streptavidin-conjugated allophycocyanin (APC) were from Caltag Laboratories, Burlingame, CA. Mouse anti-Bcl-2 PE was purchased from Santa Cruz Biotechnology, and IgG1 PE was obtained from DAKO.

Cell Preparation and FACS Analysis. The dorsal, ventral, and lateral prostates were removed from C57BL/6 mice (6 weeks old) and dissected into two regions: (*i*) the proximal region, which includes those ducts nearest the urethra, and (*ii*) the remaining region, which includes the intermediate and distal ducts. Cell digests (9) were resuspended in FACS buffer [PBS containing BSA (0.1%), sodium azide (0.01%), and aprotinin (20 μg/ml)]. Fc receptors were blocked with mouse CD16/32 antibodies and rat IgG, and the cells were incubated with antibody or control IgG for 30 min on ice and washed with FACS buffer. In some experiments, the dye 7-aminoactinomycin D (1 μg/ml) was added 5 min before analysis, so that dead cells could be excluded. Bcl-2 expression was determined in paraformaldehyde-fixed cells, permeabilized with Tween 20 (Merck-Schuchardt, Hohenbrunn, Germany). Antibodies to Sca-1, conjugated to PE, FITC, or biotin, were used in conjunction with antibodies to α6 integrin conjugated to FITC or antibodies to Bcl-2 conjugated to PE, to determine the incidence of coexpression of Sca-1 and these antigens. Cells were analyzed on a FACSCalibur flow cytometer (Becton Dickinson), using CELLQUEST software (Becton Dickinson). Sca-1$^+$ cells with fluorescent intensities in the upper one-third were defined as Sca-1^{high} cells.

Implantation of Grafts Under the Renal Capsule. Cells (1×10^5 or 3×10^4) from different regions of prostatic ducts were combined

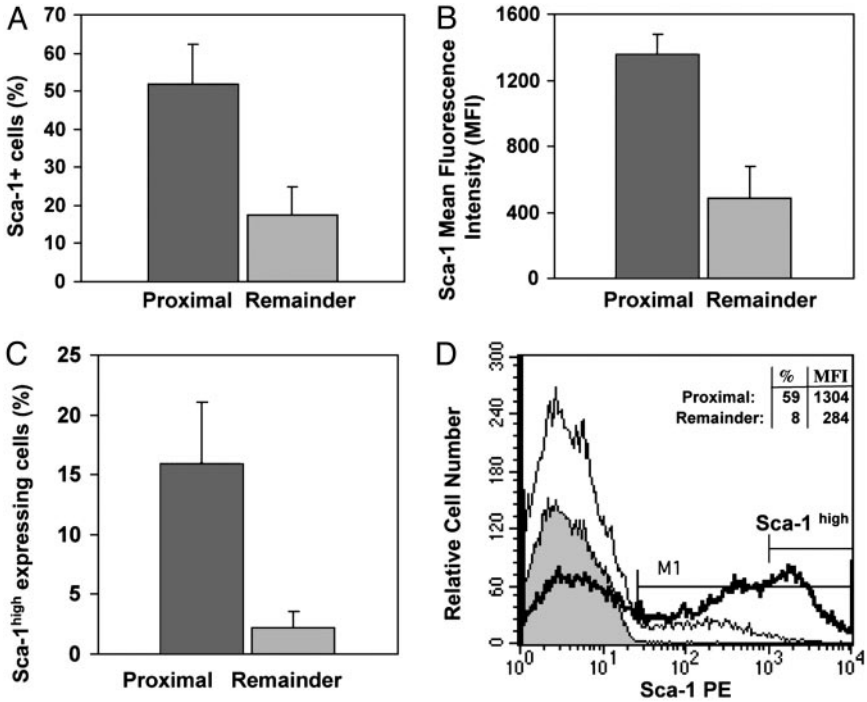

Fig. 1. Sca-1 is highly expressed by cells in the proximal region of prostatic ducts. The expression of Sca-1 by cell digests from the proximal and remaining regions of prostatic ducts was determined. (*A*) The proximal region contained 2.9-fold more Sca-1-expressing cells than the remaining ductal regions ($P < 0.0001$). (*B*) Cells from the proximal region expressed 2.8-fold more molecules of Sca-1 per cell (higher MFI) than cells from the remaining regions ($P < 0.01$). (*C*) Cells with high levels of Sca-1 expression (cells with fluorescence intensities in the upper one-third) were 7.2-fold more prevalent in the proximal region than in the remaining regions ($P < 0.00001$). Results in *A–C* are means of at least three experiments. (*D*) A representative histogram (from one of five experiments) of Sca-1 expression by viable (7-aminoactinomycin D-negative) cells from the proximal region (thick line) and the remaining regions (thin line) of ducts shows the difference in Sca-1 expression between these two regions. The gray-filled histogram represents the appropriate IgG control. The marker M1 is placed so that <1% of control cells are positive. A second marker denotes Sca-1^{high} cells.

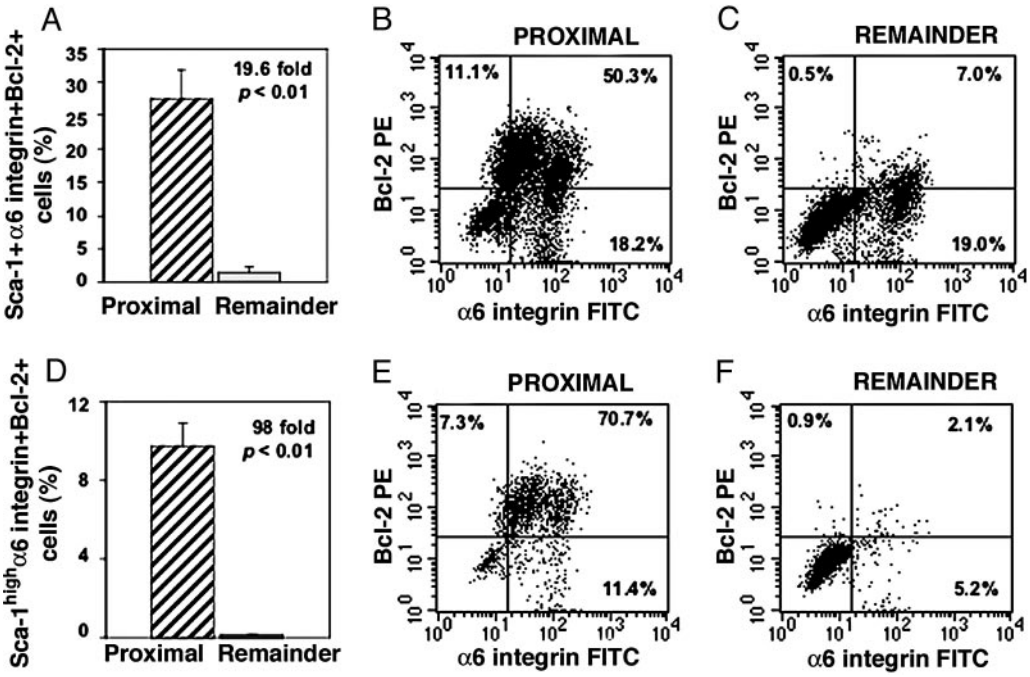

Fig. 2. The proximal region is considerably enriched in Sca-1-expressing cells that coexpress α6 integrin and Bcl-2. Three-color FACS analysis was performed to determine the incidence of Sca-1$^+$ α6 integrin$^+$ Bcl-2$^+$ cells (*A–C*) and Sca-1^{high} α6 integrin$^+$ Bcl-2$^+$ cells (*D–F*) in the proximal and remaining regions of ducts. (*A*) The proximal region contained 19.6-fold more Sca-1$^+$ α6 integrin$^+$ Bcl-2$^+$ cells than the remaining regions ($P < 0.01$). (*B* and *C*) In these representative dot plots, 50.3% of proximal Sca-1$^+$ cells coexpressed both α6 integrin and Bcl-2 (*B*), whereas 7.0% of cells from the remaining regions coexpressed these antigens (*C*). (*D*) Analysis of triple-labeled cells expressing high levels of Sca-1 showed that the proximal region contained 98-fold more Sca-1^{high} α6 integrin$^+$ Bcl-2$^+$ cells than the remaining regions ($P < 0.01$). (*E* and *F*) For these dot plots, 70% of proximal Sca-1^{high} cells coexpressed both α6 integrin and Bcl-2 (*E*), whereas 2% of cells from the remaining regions were Sca-1^{high} α6 integrin$^+$ Bcl-2$^+$ (*F*). The results are the mean of three experiments.

with urogenital sinus mesenchyme (UGM) cells (2.5×10^5) and resuspended in 30 μl of type 1 collagen (BD Biosciences). The collagen grafts were inserted under the renal capsule (23). Each experiment contained grafts of UGM alone to ensure that tissue growth did not result from contaminating urogenital sinus epithelial cells. Grafts were harvested and weighed after 8–10 weeks. UGM was isolated from the urogenital sinus of embryos (18 days old) from CDIGS rats (23–25).

Isolation of Sca-1-Expressing Cells. Prostatic duct digests were enriched for Sca-1-expressing cells by immunomagnetic separation, using magnetically activated cell sorter microbeads coated with antibodies to Sca-1 and the MiniMACS magnetically activated cell sorter system (Miltenyi Biotec, Auburn, CA). In some experiments, cells were sorted by FACS into various fractions (Sca-1^{high}, Sca-1$^{med/lo}$ or Sca-1^{neg}) according to the mean fluorescence intensity (MFI) of Sca-1 expression by the cells.

Statistical Analysis. The results are depicted as the means and standard deviations of each set of data. Comparisons between groups were made by using the two-tailed, paired Student *t* test, or in the case of different sized samples, the Mann–Whitney *U* test. A *P* value of <0.05 is considered statistically significant.

Results

Cells in the Proximal Region of Murine Prostatic Ducts Coexpress High Levels of Sca-1, α6 Integrin, and Bcl-2. We have shown that cells with stem cell features (quiescence and high proliferative potential) are concentrated in the proximal region of prostatic ducts (9). By using FACS analysis, we determined whether the expression of three antigens, Sca-1, α6 integrin, and Bcl-2, known to be expressed by stem cells of other origins (10–18, 20–22), differs between the proximal and remaining ductal regions.

We found that these three antigens are expressed by at least some cells in all regions of the ducts, but significant differences were noted in their distribution. They were expressed by a substantially higher proportion of cells in the proximal region than in the remaining regions (Table 1), and the levels of expression of each antigen (MFI) were higher in proximal cells than in cells of the remaining ductal regions. The proximal region contained a 2.9-fold ($P < 0.0001$) higher proportion of Sca-1-expressing cells that had a 2.8-fold-higher MFI ($P < 0.01$) than cells from the remaining ductal regions (Fig. 1 *A* and *B* and Table 1). Because high levels of Sca-1 are found on purified populations of other types of stem cells (14, 15, 26, 27), we determined the location of cells with high MFI for Sca-1. The proximal region of ducts contained 7.2-fold more cells with high levels of Sca-1 (Sca-1$^+$ cells with fluorescence intensities in the upper one-third) than the remaining regions (Table 1; $P < 0.00001$; Fig. 1 *C* and *D*), indicating that Sca-1^{high} cells are concentrated proximally.

Determination of the coexpression of all three antigens indicated that cells from the proximal region contain significantly more (19.6-fold; $P < 0.01$) Sca-1$^+$ α6 integrin$^+$ Bcl-2$^+$ cells (27.5 ± 4.4%) than those from the remaining regions (1.4 ± 0.8%) (Fig. 2 *A–C* and Table 1). Analysis of the proximal region for cells expressing high levels of Sca-1 together with α6 integrin and Bcl-2 (Sca-1^{high} α6 integrin$^+$ Bcl-2$^+$ cells) revealed that 98-fold more triple-labeled cells reside in the proximal compared with the other regions of ducts (9.8 ± 1.2% vs. 0.1 ± 0.06%, $P < 0.01$; Fig. 2 *D–F* and Table 1). In addition, each antigen alone was expressed by more cells (Table 1) and with a higher MFI (data not shown) in the proximal region compared with remaining regions.

These results show that there are striking differences in the distribution of cells expressing Sca-1, α6 integrin, and Bcl-2 in

different ductal regions. Cells with high levels of Sca-1 are predominantly confined to the proximal region and triple-labeled cells with high levels of Sca-1 are almost exclusively confined to this region.

Sca-1-Expressing Cells Have High *in Vivo* Proliferative Potential. The ability to regenerate tissue *in vivo* is a characteristic of stem cells, and this property has been used to identify various antigens, including Sca-1, as stem cell markers. For example, Sca-1-expressing cells isolated from bone marrow are able to reconstitute all blood cell types (10), and mammary epithelial cells enriched for Sca-1 can reconstitute the mammary gland *in vivo* and have greater growth potential than Sca-1-depleted cells (12).

We therefore determined the growth potential of Sca-1-expressing cells isolated from the proximal and the remaining ductal regions and compared their proliferative potential *in vivo* with cells that did not express this antigen. Sca-1-expressing (Sca-1^+) and Sca-1-depleted (Sca-1^-) populations were isolated from digests of the proximal and the remaining ductal regions by using antibodies to Sca-1 and magnetic microbeads. These populations were combined with cells isolated from the UGM [inductive mesenchyme for prostatic tissue (23–25)] and inserted under the renal capsule of recipient male animals, and the amount of prostatic tissue generated was measured after 8 weeks. Sca-1^+ cells isolated from the proximal region formed significantly more prostatic tissue (203.0 ± 83.1 mg; 17.1-fold) than was obtained from the Sca-1^- proximal population (11.9 ± 9.2 mg; $P < 0.001$) (Fig. 3 *A* and *B*). Sca-1^+ cells isolated from the remaining ductal regions also formed prostatic tissue under the renal capsule (31.9 ± 24.1 mg) but formed far less tissue than observed for Sca-1^+ cells isolated from the proximal region (203.0 ± 83.1 mg), indicating that these two Sca-1^+ populations differ markedly in their *in vivo* growth potential ($P < 0.001$). Sca-1^- cells isolated from the remaining regions of ducts formed very little subrenal capsule tissue (6.6 ± 5.0 mg).

Because our FACS data showed that cells expressing high levels of Sca-1 were confined predominantly to the proximal region of ducts, we determined whether Sca-1^{high} cells had greater growth potential than cells with medium/low Sca-1 expression. Proximal cell populations were FACS-sorted into fractions containing cells with high MFI (Sca-1^{high}), medium to low MFI (Sca-$1^{med/lo}$) and no Sca-1 expression (Sca-1^{neg}) and inserted under the renal capsule of recipient animals. Sca-1^{high} cells formed significantly more prostatic tissue (43.7 ± 26.8 mg; 6.3-fold) than Sca-$1^{med/lo}$ cells (6.9 ± 3.6 mg; $P < 0.001$) and 7.5-fold more tissue than Sca-1^{neg} cells (5.8 ± 1.6 mg; $P = 0.001$) (Fig. 4 *A–C*). Although the tissue growth obtained was less than in experiments using magnetic beads (because of the stress that the cells undergo during FACS sorting), these results show that almost all of the *in vivo* growth potential is confined to cells that express high levels of Sca-1. The prostatic tissue obtained from Sca-1^{high} cells had normal prostatic histology comprising basal and luminal cells lining prostatic ducts. The lumens of the ducts contained abundant amounts of secretory material (Fig. 4*C*). In contrast, the tissue arising from Sca-$1^{med/lo}$ and Sca-1^{neg} cells contained more stroma with less of an epithelial component, and little secretory material was generally noted within the ducts (Fig. 4*C*).

These results show that cells expressing Sca-1 have considerably more growth potential than those lacking this antigen, and that the proliferative ability within the Sca-1-expressing proximal cells resides in cells that express high levels of this antigen. They also show that Sca-1-expressing cells residing in the proximal region are more primitive than those Sca-1-expressing cells in the remaining ductal regions, as they have far higher proliferative capacity. These data indicate that stem cells reside within the Sca-1-expressing population in the proximal region, whereas the transit-amplifying cells, with more limited growth potential, reside within the Sca-1-expressing cells in the remaining ductal regions.

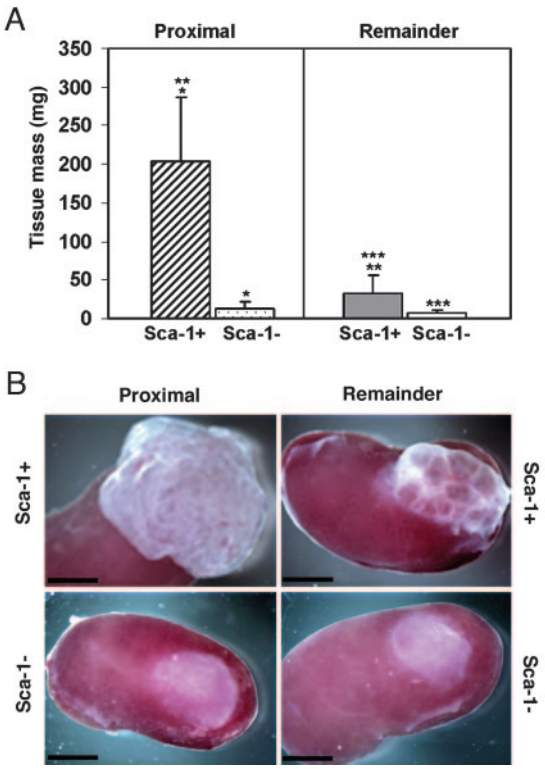

Fig. 3. Sca-1^+ cells have greater *in vivo* proliferative capacity than Sca-1^- cells. (*A*) The growth of Sca-1^+ and Sca-1^- cells (10^5 cells) that were isolated from either the proximal region or the remaining regions of ducts and transplanted under the renal capsule was measured after 8 weeks. Sca-1^+ cells obtained from the proximal region formed 17.1-fold more prostatic tissue than Sca-1^- cells (*, $P < 0.001$). Sca-1^+ cells obtained from the remaining ductal regions had far less growth potential than Sca-1^+ proximal cells (**, $P < 0.001$). Sca-1^- cells from the remaining regions showed less growth than Sca-1^+ cells from this region (***, $P < 0.001$). The results are the means of two experiments, using the data obtained from the inoculation of a total of 7, 11, 14, and 12 kidneys with Sca-1^+ and Sca-1^- cells from the proximal and remaining regions, respectively. (*B*) Prostate tissue under the renal capsule initiated with 10^5 Sca-1^+ or Sca-1^- cells from either the proximal region or the remaining regions. (Scale bars: 3 mm.)

Discussion

We show that Sca-1-expressing cells, isolated from the proximal region of ducts, form significantly more prostatic tissue *in vivo* than cells that lack this antigen and that most of the proliferative ability resides in cells that express high levels of this antigen. In addition, Sca-1-expressing cells isolated from the proximal region have far greater proliferative potential than Sca-1-expressing cells isolated from the remaining ductal regions (which express lower levels of Sca-1 per cell than proximal cells). The Sca-1^{high} cells that coexpress α6 integrin and Bcl-2 are almost exclusively confined to the proximal region of ducts, indicating that prostate stem cells express high levels of Sca-1 together with α6 integrin and Bcl-2.

Sca-1 is expressed by stem cells from a variety of origins including hematopoietic tissue, heart, mammary gland, skin, muscle, and testis (10–15). Although a ligand for Sca-1 has not been identified, Sca-1 appears to be important for the self-renewal of mesenchymal (28) and hematopoietic (29) stem cells. Sca-$1^{-/-}$ mice have greatly reduced bone mass resulting from a primary defect in the self-renewal capacity of early mesenchymal progenitor cells (28). In addition, hematopoietic stem cells from Sca-$1^{-/-}$ mice have a

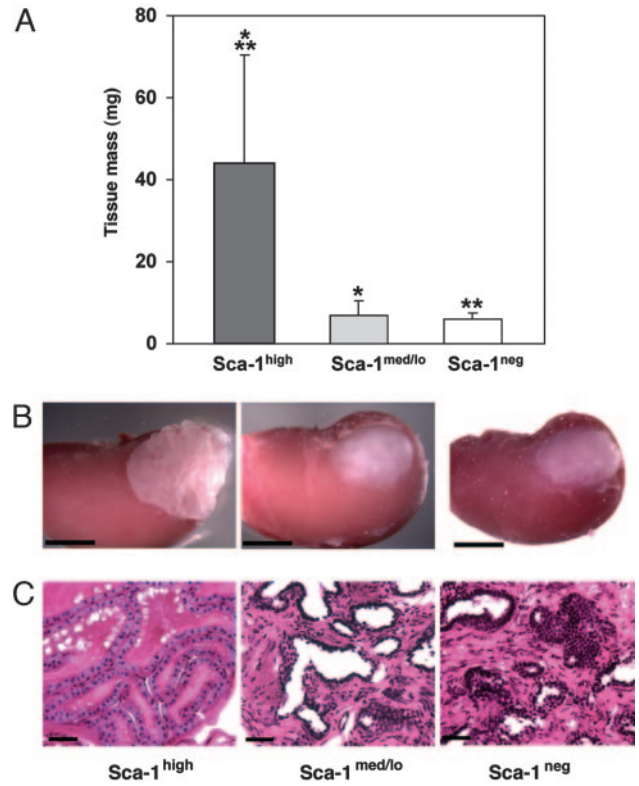

Fig. 4. Sca-1^{high} cells have greater *in vivo* proliferative capacity than cells that express lower levels of Sca-1. Cells were isolated from the proximal region and sorted by FACS into Sca-1^{high}, Sca-1$^{med/lo}$ and Sca-1^{neg} fractions according to the level of Sca-1 expression. The cell populations (3×10^4) were transplanted under the renal capsule and the growth of prostatic tissue was measured after 10 weeks. (*A*) Sca-1^{high} cells formed 6.3-fold more prostatic tissue than Sca-1$^{med/lo}$ cells (*, $P <$ 0.001) and 7.5-fold more prostatic tissue than Sca-1^{neg} cells (**, $P = 0.001$). These results are the means of two experiments, using the data obtained from the inoculation of a total of 10, 9, and 10 kidneys with cell populations containing Sca-1^{high}, Sca-1$^{med/lo}$ and Sca-1^{neg} cells, respectively. (*B*) Prostate tissue initiated with 3×10^4 Sca-1^{high}, Sca-1$^{med/lo}$, or Sca-1^{neg} cells isolated by FACS from the proximal region of ducts. (Scale bars: 3 mm.) (*C*) Paraffin sections stained with hematoxylin and eosin showing the morphology of prostatic tissue arising from Sca-1^{high}, Sca-1$^{med/lo}$ or Sca-1^{neg} cells. The prostatic tissue obtained from Sca-1^{high} cells had normal prostatic histology comprising basal and luminal cells lining prostatic ducts. The lumens of the ducts were filled with secretory material. The tissue arising from Sca-1$^{med/lo}$ and Sca-1^{neg} cells contained increased stroma with less of an epithelial component, and little secretory material was noted within the ducts. (Scale bars: 40 μm.)

decreased repopulation potential and manifest a lower engraftment of secondary transplants than cells from wild-type mice (29), indicating that Sca-1 is required for self-renewal. These findings are consistent with our data showing that Sca-1^{neg} cells have little capacity to generate prostatic tissue when implanted under the renal capsule, and indicate that Sca-1 may also be involved in the self-renewal of stem cells in the prostate.

Stem cells are rare cells, and, because large numbers of cells isolated from prostatic ducts express Sca-1, it is unlikely that all Sca-1-expressing cells are stem cells. Our data, in fact, indicate that prostatic stem cells reside in the Sca-1^{high} population that also expresses α6 integrin and Bcl-2. The presence of α6 integrin together with high levels of Sca-1 is also characteristic of spermatogonial stem cells (15). Stem cells from other origins also express α6 integrin. The gene for this integrin was the only common gene identified in a study using transcriptional profiling to identify genes expressed by stem cells of embryonic, neural, hematopoietic, and retinal origin (30). Keratinocyte stem cells also express high levels of α6 integrin (31), and these cells have enhanced long-term proliferative potential (32).

Members of the integrin family are important regulators of stem cell function (33). Keratinocyte and putative prostate stem cells are more adhesive than the more mature transit-amplifying cells, and putative human prostate stem cells express high levels of α2 integrin (34–36). It is possible that a number of members of the integrin family are expressed by stem cells because there is recent evidence that the adhesive properties of integrins may be involved in maintaining stem cells within their niche (37, 38). Because stem cells and cancer cells have many similar properties (1–5), it is of interest that changes in the expression of integrins, particularly $\alpha 6\beta 4$ integrin, are implicated in tumorigenesis and invasion and that the α6 integrins play a role in the progression of cancer (39–41).

The prostate cells from the proximal region that express high levels of Sca-1 also coexpress the antigen Bcl-2. The presence of Bcl-2 in Sca-1-expressing prostate stem cells may protect these cells from apoptotic death. Stem cells are needed for the lifetime of their host, and mechanisms to protect them from death are important to ensure their long-term survival. The Bcl-2 protein suppresses apoptosis (19) and is present in many long-lived cells (42). Bcl-2 protects hematopoietic and keratinocyte stem cells from apoptotic death (21, 43), and over-expression of Bcl-2 increases the numbers of hematopoietic stem cells *in vivo* (20) and protects hematopoietic stem cells from the harmful effects of a number of chemotherapeutic agents, thus ensuring their survival (44). The expression of Bcl-2 by the prostate stem cell population that has high levels of Sca-1 and significant *in vivo* proliferative potential is therefore likely to ensure the long-term survival of this cell population.

High levels of Bcl-2 in the proximal stem cell region may also be required to protect these cells from apoptosis that accompanies androgen withdrawal. Castration results in an increase in TGF-β levels (45), leading to apoptosis and involution of the more distal regions of the gland, whereas the proximal region is relatively unchanged (46, 47). We find a TGF-β signaling gradient in prostatic ducts, with high levels of signaling in the quiescent proximal region (high Bcl-2 expression) and low levels of signaling in the distal region (low Bcl-2 expression) (S.N.S., P.E.B., S.C., K.G., D.M., and E.L.W., unpublished data). The proximal region is therefore protected from TGF-β-mediated apoptosis by high Bcl-2 expression. Aberrant regulation of Bcl-2 expression may contribute to the etiology of prostatic diseases such as benign prostatic hyperplasia (48), proliferative inflammatory atrophy, which is a regenerative lesion that may give rise to prostate cancer (49), and prostate cancer itself (50). In addition, the over-expression of Bcl-2 is implicated in the formation of hormone-independent prostate tumors because it inhibits the apoptotic effect of TGF-β and androgens (51). The identification of the phenotype of prostatic stem cells that express high levels of Bcl-2 may therefore aid in identifying the target cells from which these lesions originate.

The identification of other antigens expressed by the population of cells that express Sca-1, α6 integrin, and Bcl-2 may result in the definition of a more comprehensive phenotype for prostate stem cells. For example, the expression of antigens such as CD133 (prominin), which has been found on human putative prostatic stem cells (52), signaling molecules, such as Wnt, Notch, and Hedgehog, that are involved in stem cell renewal and maintaining stem cell niches (53, 54), and members of the Polycomb family, such as Bmi1 and EZH2 (55, 56), may further stratify the prostatic stem cell phenotype. Because cancers may arise from mutations in stem cells (2, 4, 5) and because benign prostatic hyperplasia may result from aberrant proliferation of these cells (7), the identification of the stem cell phenotype of prostate cells may permit the development of rational targeted therapies for treating both conditions.

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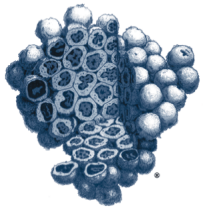

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Ken Goto, Sarah N. Salm, Sandra Coetzee, Xiaozhong Xiong, Patricia E. Burger, Ellen Shapiro, Herbert Lepor, David Moscatelli and E. Lynette Wilson

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Proximal Prostatic Stem Cells Are Programmed to Regenerate a Proximal-Distal Ductal Axis

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Key Words. Proximal prostate stem cells • Ductal axis • Androgen sensitivity

ABSTRACT

Prostate carcinoma and benign prostatic hypertrophy may both originate in stem cells, highlighting the importance of the characterization of these cells. The prostate gland contains a network of ducts each of which consists of a proximal (adjacent to the urethra), an intermediate, and a distal region. Here, we report that two populations of cells capable of regenerating prostatic tissue in an in vivo prostate reconstitution assay are present in different regions of prostatic ducts. The first population (with considerable growth potential) resides in the proximal region of ducts and in the urethra, and the survival of these cells does not require the presence of androgens. The second population (with more limited growth potential) is found in the remaining ductal regions and requires androgen for survival. In addition, we find that primitive proximal prostate cells that are able to regenerate functional prostatic tissue in vivo are also programmed to re-establish a proximal-distal ductal axis. Similar to their localization in the intact prostate, cells with the highest regenerative capacity are found in the proximal region of prostatic ducts formed in an in vivo prostate reconstitution assay. The primitive proximal cells can be passaged through four generations of subrenal capsule grafts. Together, these novel findings illustrate features of primitive prostate cells that may have implications for the development of therapies for treating proliferative prostatic diseases. STEM CELLS *2006;24:1859–1868*

INTRODUCTION

The rodent prostate is an androgen-dependent organ with a ductal system that displays significant heterogeneity along the proximal-distal ductal axis. The different regions of prostatic ducts are heterogeneous in terms of morphology, telomerase expression, and levels of active transforming growth factor (TGF)-β signaling [1–3]. We have previously shown that the proximal region of mouse prostatic ducts is enriched in cells exhibiting stem cell-like properties, namely quiescence, a high proliferative potential, and the ability of single cells to generate progeny of more than one lineage [4]. These cells express Sca-1 [5, 6] and are maintained in a quiescent state by high levels of TGF-β [3]. Stem cells and tumor cells have many similar features, including infinite lifespan, self-renewal, multi-drug resistance, telomerase expression, and in the instance of the prostate, androgen independence. Evidence supports a role for stem cells in the etiology of many types of cancer [7–12]. The evolution of androgen-independent prostate carcinoma may reflect the emergence of stem-like prostate tumor cells. The distribution of androgen-independent cells within the ductal regions of a normal prostate gland is currently unknown. The investigation of regional sensitivities to androgens may increase our understanding of both normal prostate physiology and the aberrant proliferation that occurs in prostatic diseases such as benign prostatic hypertrophy and prostate carcinoma.

We therefore isolated cells from different regions of prostatic ducts removed from donor androgen-replete or castrated animals and examined their ability to regenerate prostatic tissue in recipient androgen-replete as well as in androgen-ablated and reconstituted animals. This was done using an in vivo prostate reconstitution assay in which combinations of prostate cells and embryonic urogenital sinus mesenchyme (UGM)

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(inductive mesenchyme for prostatic tissue) [13–15] are inserted under the renal capsule (RC) of recipient animals, where they form prostatic tissue. We find that cells isolated from the intermediate and distal regions do not survive androgen ablation whereas those from the proximal region and the urethra are able to withstand prolonged androgen deprivation, indicating the presence of cells with stem cell properties in these areas. Additionally, we show for the first time that primitive proximal prostate cells, which regenerate functional prostatic tissue, are programmed to re-establish a proximal-distal ductal axis when inserted under the RC. Similar to the primitive cells in the intact prostate, the cells with the highest regenerative capacity are also found in the proximal region of ducts in the sub-RC prostatic tissue and can be passaged through four generations of sub-RC grafts.

Materials and Methods

Animals

C57BL/6 mice (Taconic, Germantown, NY, http://www.taconic.com), athymic nude mice (National Institutes of Health, Bethesda, MD, http://www.nih.gov), CDIGS rats (Charles River Laboratories, Wilmington, MA, http://www.criver.com), and green fluorescent protein (GFP) transgenic mice (C57BL/6-TgN; The Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) were housed in a climate-controlled facility, and all animal care and procedures were performed in compliance with the New York University institutional review board requirements.

Preparation of Dissociated Prostate and Urethra Cells

Six-week-old C57BL/6 mice were sacrificed, and the urogenital tract was removed en bloc and transferred in Hanks' balanced salt solution (Mediatech, Inc., Herndon, VA, http://www.cellgro.com). The dorsal prostate (DP) was removed and dissected under a dissecting microscope in the presence of 0.5% collagenase (1.3 units/mg; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) [16]. The proximal, intermediate, and distal regions (Fig. 1AA) were excised, minced finely, and incubated in collagenase for 60 minutes followed by digestion in 0.25% trypsin (DIFCO Bacto Trypsin 250; BD Biosciences, Sparks, MD, http://www.bd.com) for an additional 10 minutes at 37°C [4]. A portion of the urethra was removed (Fig. 1A) and was similarly digested. Cells were passed through a 40-μm nylon mesh (Becton, Dickinson and Company, Franklin Lakes, NJ, http://www.bd.com), and viability was determined by trypan blue exclusion.

Preparation of UGM Cells

UGM was isolated from the urogenital sinus of 18-day-old CDIGS rat embryos after digestion with trypsin (1%) at 4°C for 90 minutes [13, 14]. A single-cell suspension of UGM cells was obtained by digesting the UGM tissue for 10 minutes at 37°C in collagenase (0.5%). Fetal rat UGM was used in place of fetal mouse UGM because rat UGM promotes growth more effectively than does mouse UGM [14].

Implantation of Grafts Under the RC

The grafts were implanted under the RC of intact or castrated athymic male mice [13] (tutorial for technique: http://mmmary.nih.gov/tools/Cunha001/index.html). Cells from the urethra or different regions of ducts (10^5 unless otherwise indicated) were combined with UGM cells (2.5×10^5) and resuspended in 15 μl of type 1 collagen (BD 354236; BD Biosciences, Bedford, MA,

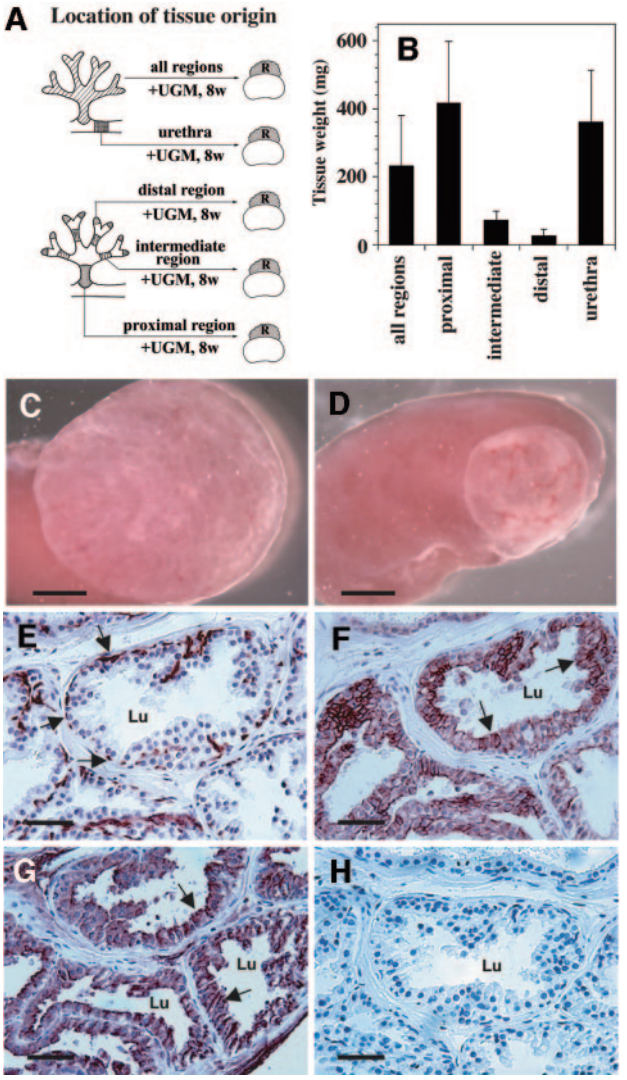

Figure 1. The proximal region of mouse prostatic ducts and the urethra contain stem cells. **(A):** Schematic diagram of the prostate showing the protocol used to implant different regions of prostatic ducts under the RC. **(B):** Cells from the urethra ($n = 15$) or different regions of ducts (10^5) (all regions $n = 14$, proximal $n = 37$, intermediate $n = 8$, distal $n = 26$) were combined with UGM cells (2.5×10^5) and implanted under the RC. Grafts were harvested after 8 weeks, weighed, and used for immunocytochemical examination. Each bar represents the mean ± SD. **(C):** Prostatic tissue under the RC initiated with 10^5 proximal cells. Bar = 2 mm. **(D):** Prostatic tissue under the RC initiated with 10^5 distal cells. Bar = 2 mm. **(E):** A section of prostatic tissue arising from proximal cells showing basal cells (arrows) immunohistochemically stained using an antibody against K5 keratin. Bar = 40 μm. **(F):** A section of prostatic tissue arising from proximal cells showing luminal cells (arrows) immunohistochemically stained using an antibody against K8 keratin. Bar = 40 μm. **(G):** A section of prostatic tissue arising from proximal cells immunohistochemically stained with antibodies specific for prostatic secretory products (arrows). Bar = 40 μm. **(H):** A section of proximal prostatic tissue indicating no staining in tissues to which appropriate control antibodies were added, showing that staining is specific. Bar = 40 μm. Abbreviations: Lu, lumen of duct; RC, renal capsule; UGM, urogenital sinus mesenchyme.

http://www.bdbiosciences.com). The collagen was allowed to gel at 37°C for 15 minutes after which the grafts were inserted under the RC. Where indicated, androgens were administered by the subcutaneous implantation of testosterone pellets (5 mg; Innovative Research of America, Sarasota, FL, http://www.innovrsrch.com). Each experiment contained a set of grafts of UGM alone (3.5×10^5 cells) to ensure that tissue growth did not result from contaminating urogenital sinus epithelial cells. In addition, some experiments were done using prostate cells isolated from GFP transgenic mice (C57BL/6-TgN; The Jackson Laboratory) to ensure that tissue growth resulted from donor GFP-expressing cells and not contaminating epithelial cells in the UGM preparation (supplemental online Fig. 3). Grafts were harvested after 8 weeks of in vivo growth, weighed, and used for immunohistochemical examination.

Passage of Undissected Recombinant Tissue (All Regions) In Vivo

The ability of proximal and distal regions of primary prostate cells to undergo multiple rounds of growth was assessed by serial in vivo passaging of recombinant tissue. Cells isolated from proximal and distal regions of prostatic ducts (1×10^5) were combined with UGM (2.5×10^5 cells) and implanted under the RC of intact 6-week-old male athymic nude mice. After 8 weeks, the recipient mice were sacrificed, and grafts from either proximal or distal cells (P1) (Fig. 2A) were retrieved and weighed. Grafts arising from either proximal or distal cells were minced finely, digested in collagenase (see above), and trypan blue-excluding cells were enumerated. These cells (1×10^5 cells) were combined with UGM (2.5×10^5 cells) and implanted into recipient mice to produce a "second passage (P2)" graft (Fig. 2A). This protocol was repeated until no tissue growth was noted.

Passage of the Proximal Region of Recombinant Tissue In Vivo

Sub-RC grafts from cells isolated from the proximal region were digested with collagenase (see above), revealing a ductal network similar to that observed in a prostate removed from an animal (P1; Fig. 3B, 3C). To determine whether the sub-RC grafts maintained a proximal-distal axis and to ascertain whether cells within the proximal and distal regions of these grafts exhibited the differential growth capacity of proximal and distal cells isolated from a "primary" prostate (Fig. 3A), we dissected the recombinant tissue arising from proximal cells into proximal and distal regions. Single-cell suspensions of these regions were prepared (see above), and proximal and distal cells (1×10^5) were each combined with UGM (2.5×10^5 cells) and implanted into a second generation of recipient animals to produce a "P2" graft (Fig. 3A). The P2 graft arising from proximal cells was again dissected into proximal and distal regions and passaged as above into a third generation of recipient animals (P3, Fig. 3A). This protocol was repeated until no tissue growth was observed (Fig. 3A). After each tissue passage, animals were sacrificed after 8 weeks of in vivo growth and grafts were removed and weighed.

Immunohistochemistry

Grafts were fixed in 70% ethanol or 3% paraformaldehyde and embedded in paraffin, and sections were stained with hematoxylin and eosin. Immunohistochemistry was performed as

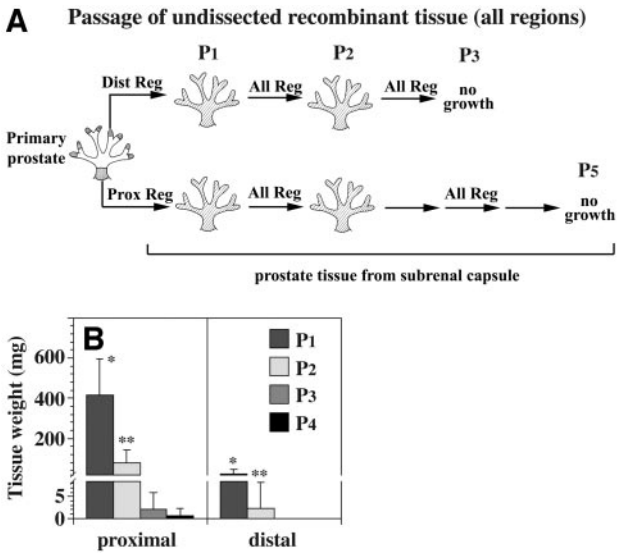

Figure 2. Cells from the proximal region of primary prostate tissue can be serially passaged in vivo. **(A):** Schematic diagram showing the protocol used for passaging proximal and distal cells isolated from primary prostate tissue. Proximal (prox reg) and distal (dist reg) cells (10^5) were combined with UGM cells (2.5×10^5) and implanted under the RC of intact animals. Grafts arising from proximal and distal cells were harvested after 8 weeks, the entire graft (all reg) was digested with collagenase and trypsin, and cells (10^5) from each type of graft were again combined with UGM cells (2.5×10^5) and implanted under the RC for an additional 8 weeks. This process was repeated until no further tissue growth was noted. **(B):** The tissue arising from proximal and distal cells at each passage (P1–P4) was weighed. Proximal cells: P1 $n = 37$, P2 $n = 8$, P3 $n = 8$, P4 $n = 7$. Distal cells: P1 $n = 26$, P2 $n = 7$. P1, proximal versus distal: *, $p < .001$; P2, proximal versus distal: **, $p < .001$. Each bar represents the mean ± SD. Abbreviations: RC, renal capsule; UGM, urogenital sinus mesenchyme.

described previously [17, 18]. Mouse monoclonal antibodies to cytokeratin-8 (RDI-PRO65138; Research Diagnostics, Inc., Flanders, NJ, http://www.researchd.com) and α-smooth muscle actin (A2547; Sigma-Aldrich) were directly coupled to horseradish peroxidase (HRP) using Dako's Envision + system (Dako, Carpinteria, CA, http://www.dako.com) and detected using DAB (3,3′ diaminobenzidine) as the substrate. Rabbit polyclonal antibodies that are specific to Nkx3.1 [19] and to secretions of the DP were a gift of Dr. C. Abate-Shen (Robert Wood Johnson Medical School, Piscataway, NJ; M. Kim, M.M. Shen, and C. Abate-Shen, personal communication) [4]. Cytokeratin-5 was visualized using rabbit polyclonal antibodies (PRB-160P; Covance, Princeton, NJ, http://www.covance.com) and appropriate HRP-linked secondary antibodies (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com). GFP was visualized using rabbit polyclonal antibodies (46–0092; Invitrogen, Carlsbad, CA, http://www.invitrogen.com) and the ABC staining kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com). Androgen receptors were visualized using rabbit polyclonal antibodies (sc-816; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, http://www.scbt.com) and the ABC staining kit (Vector Laboratories). The specificity of staining was ascertained on sections using nonimmune serum or immunoglobulin G in place of primary antibodies. Sections were counterstained with hematoxylin.

Isolation of α6 Integrin Expressing Cells

Samples were enriched for α6 integrin (CD49f)-expressing cells by immunomagnetic separation using antibodies to this antigen (BD 555734; BD Biosciences) and magnetically activated cell sorter (MACS) microbeads, magnetic columns, and the Mini-MACS system (Miltenyi Biotec, Auburn, CA, http://www.miltenyibiotec.com).

Cell Preparation and Fluorescence-Activated Cell Sorting Analysis

Prostatic cell digests obtained from either the proximal region or the remaining (intermediate and distal) regions were resuspended in fluorescence-activated cell sorting (FACS) buffer (phosphate-buffered saline containing bovine serum albumin [0.1%], sodium azide [0.01%], and aprotinin [20 μg/ml]) [5]. For analysis of viable α6 integrin-expressing cells, the dye 7-aminoactinomycin D (1 μg/ml) was added 5 minutes prior to FACS acquisition to cells treated with antibodies to α6 integrin conjugated to fluorescein isothiocyanate (FITC). Analysis of the co-expression of Sca-1, α6 integrin, and Bcl-2 was determined in permeabilized paraformaldehyde-fixed cells using antibodies to α6 integrin conjugated to FITC (BD 555735; BD Biosciences) in conjunction with antibodies to Bcl-2 conjugated to phycoerythrin (PE) (sc-7382 PE; Santa Cruz Biotechnology, Inc.) and antibodies to Sca-1 conjugated to biotin (MSCA15; Caltag Laboratories, Burlingame, CA, http://www.caltag.com) followed by streptavidin APC (SA1005; Caltag Laboratories) [5]. Cells were analyzed on a FACSCalibur flow cytometer (Becton, Dickinson and Company), using CellQuest software (Becton, Dickinson and Company).

Statistical Analysis

The results are depicted as the means and SDs of each set of data. Comparisons between groups were made using the two-tailed, paired Student's t test, or the nonparametric two-tailed Mann-Whitney U test. A p value of $< .05$ was considered statistically significant.

Results

Single Cell Populations of Proximal and Urethral Cells Form Large Amounts of Prostatic Tissue Under the RC

The mouse prostate can be divided into ventral, dorsal, and lateral lobes, each of which contains an arborizing network of ducts that consists of a proximal (adjacent to the urethra), an intermediate, and a distal region [3, 4, 16, 20] (Fig. 1A). We first determined the ability of cells from different regions to regenerate the prostate in androgen-replete animals. Cells (10^5) isolated from each region of the DP, from a pool of all regions together and from the urethra, were combined with UGM cells (2.5×10^5) and their proliferative capacities determined by measuring the size of tissue grafts 8 weeks after implantation under the RC (Fig. 1A, 1B). The urethra was examined because this epithelium has previously been shown to form prostatic tissue when combined with UGM [21] and we wished to determine its regenerative capacity relative to cells isolated from different regions of prostatic ducts. Cells isolated from the proximal region of ducts formed significantly more prostatic tissue (417 ± 180 mg) than did cells isolated from the

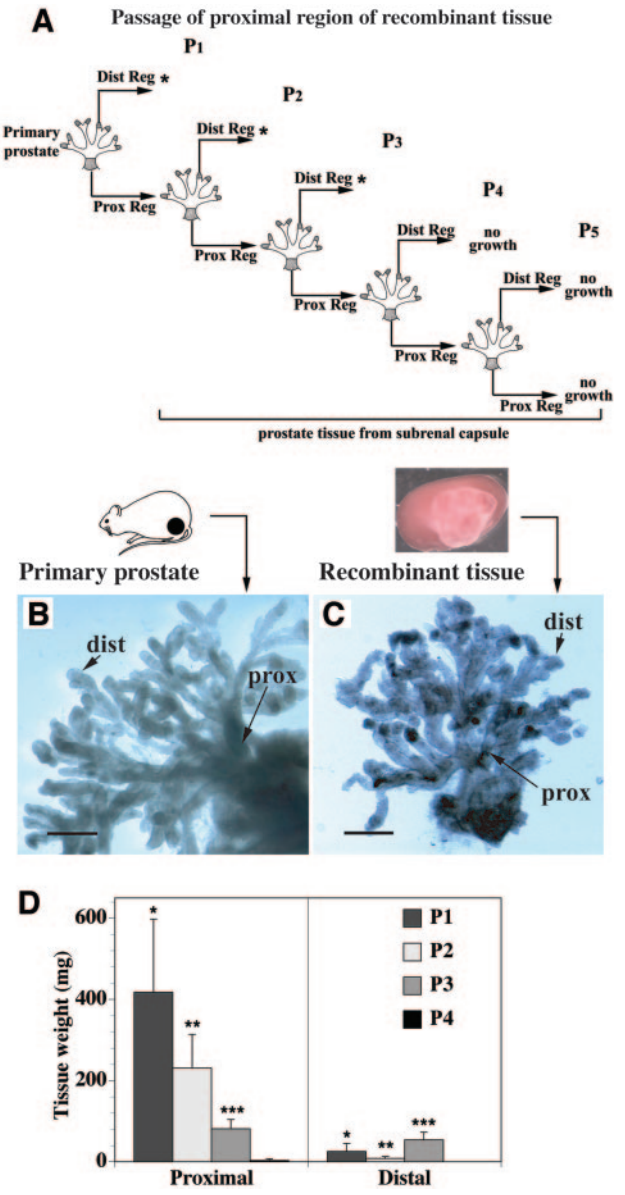

Figure 3. Cells from the proximal region of recombinant tissue can be serially passaged in vivo. **(A):** Schematic diagram showing the protocol used for passaging proximal (prox reg) and distal (dist reg) cells isolated from recombinant tissue. Proximal and distal cells (10^5) were isolated after collagenase/trypsin digestion of successive passages of sub-RC tissue, combined with UGM cells (2.5×10^5) and implanted under the RC of intact animals until no further tissue growth was noted. Asterisk denotes minimal tissue growth. **(B):** The morphology of the prostatic ductal system of a collagenase-digested lobe of the dorsal prostate showing the prox and dist regions of ducts. Bar = 0.5 mm. **(C):** The morphology of the prostatic ductal system of collagenase-digested recombinant prostate tissue arising from proximal cells showing the prox and dist regions of ducts. This indicates that the recombinant tissue has the same morphology and proximal-distal ductal axis as a primary prostate. Bar = 0.5 mm. **(D):** The tissue arising from each passage of proximal and distal cells obtained from the recombinant tissue was weighed at each successive passage (P1–P4). Proximal cells: P1 $n = 37$, P2 $n = 4$, P3 $n = 7$, P4 $n = 6$. Distal cells: P1 $n = 26$, P2 $n = 5$, P3 $n = 6$, P4 $n = 4$. P1, proximal versus distal: *, $p < .001$; P2, proximal versus distal: **, $p < .02$; P3, proximal versus distal: ***, $p < .04$. Each bar represents the mean ± SD. Abbreviations: dist, distal; prox, proximal; RC, renal capsule; UGM, urogenital sinus mesenchyme.

intermediate (65 ± 27 mg) or distal regions (25 ± 19 mg) ($p < .001$), indicating that the cells with the greatest regenerative potential reside in the proximal region. Comparable amounts of prostatic tissue were also obtained from urethral cells. Cells from the proximal region formed 17-fold more tissue than did cells from the distal region (Fig. 1B–1D) and gave rise to prostatic tissue that is 38-fold larger than that of a normal prostate gland in situ (11.0 ± 1.1 mg for DP). Similar results were obtained with cells isolated from the ventral prostate (VP), with proximal cells from this gland forming 180 ± 45 mg of tissue whereas cells from the distal region formed 11 ± 3 mg. Histological analysis revealed a complex ductal network comparable with that of a normal prostate, containing basal (Fig. 1E) and luminal (Fig. 1F) cells with secretory material of prostatic origin (Fig. 1G) in the luminal cells. The epithelial cells expressed the Nkx3.1 protein, (supplemental online Fig. 1), indicating that they were of prostatic origin as the expression of Nkx3.1 is prostate-specific [22]. Androgen receptors were also evident in the sub-RC prostatic tissue (supplemental online Fig. 2). The histological appearance of tissue arising from cells isolated from different regions was similar.

Each experiment contained grafts consisting of UGM alone to ensure that tissue growth did not result from contaminating urogenital sinus epithelial cells. The UGM grafts were weighed and examined microscopically to exclude urogenital sinus epithelial contamination. The average weight of UGM tissue alone was 8.3 ± 2.2 mg. In addition, some experiments were done using prostate cells isolated from GFP transgenic mice to ensure that tissue growth resulted from donor GFP-expressing cells and not contaminating epithelial cells in the UGM preparation (supplemental online Fig. 3).

To determine the minimum number of cells capable of forming prostatic tissue, the sub-RC growth of varying numbers of proximal and distal cells (10^5 to 4×10^2 cells) was determined (supplemental online Fig. 4). The proximal region contained cells with 50-fold greater regenerative capacity than those from the distal region as 400 proximal cells formed prostatic tissue whereas 20,000 distal cells were required for tissue growth. A linear relationship between the tissue mass and inoculum dose was noted between 400 and 10^5 cells (supplemental online Fig. 4). The size of the UGM inoculum also affected tissue size with a linear relationship in its ability to support prostate tissue growth between 2.5×10^4 and 2.5×10^5 UGM cells (data not shown).

These results indicate that isolated cells from the proximal region of ducts as well as urethral cells have considerably greater in vivo regenerative potential than do cells from other ductal regions.

Cells from the Proximal Region and the Urethra Survive Prolonged Androgen Deprivation

Because prostatic tissue can regenerate after androgen withdrawal and replenishment, we reasoned that cells from regions most enriched in androgen-independent cells having regenerative potential would survive prolonged androgen deprivation and would regenerate prostatic tissue to a greater extent than cells isolated from regions that consisted mainly of transit-amplifying cells. Transit-amplifying cells are considered to be progenitor cells that are capable of division and that represent a post-stem cell compartment.

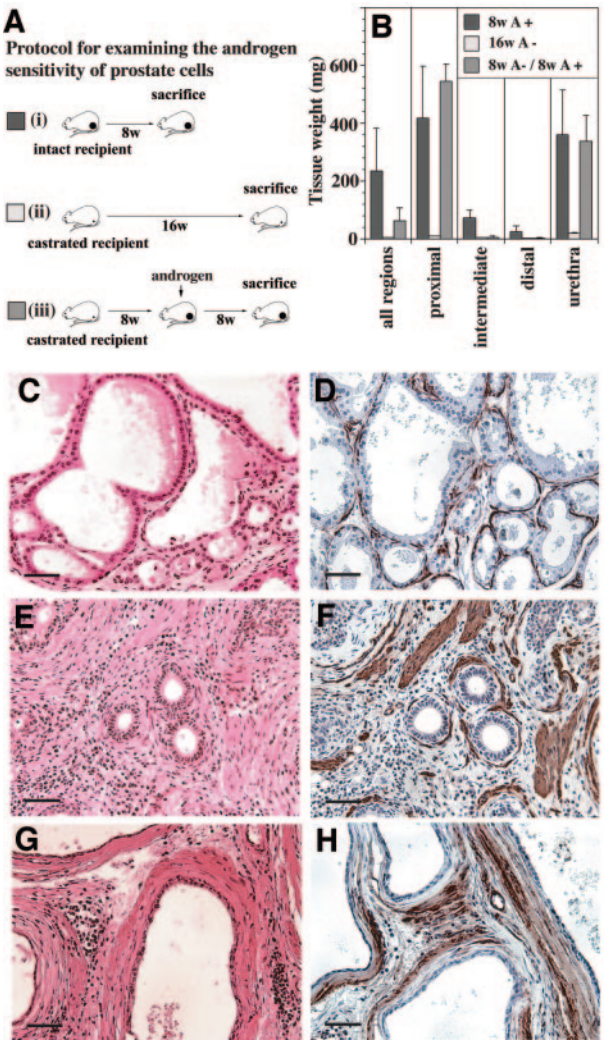

Figure 4. Cells from the proximal region and the urethra survive prolonged androgen deprivation. **(A):** Schematic diagram showing the protocol used for examining the androgen sensitivity of prostate cells. **(B):** Cells from the urethra ($n = 15$) or different regions of ducts (10^5) (all regions $n = 14$, proximal $n = 37$, intermediate $n = 8$, distal $n = 26$) were combined with UGM cells (2.5×10^5) and implanted under the RC of intact animals (8w A+), castrated animals (16w A−) (all regions $n = 3$, proximal $n = 3$, intermediate $n = 3$, distal $n = 5$, urethra $n = 2$), or animals that had been castrated for 8 weeks followed by androgen supplementation for 8 weeks (8w A−/8w A+) (all regions $n = 5$, proximal $n = 4$, intermediate $n = 6$, distal $n = 4$, urethra $n = 4$). Grafts were harvested at the indicated times (**A**), weighed, and used for immunocytochemical examination. Each bar represents the mean ± SD. **(C, E, G):** Sections of prostate tissue from intact **(C)**, castrated **(E)**, and castrated and androgen-replenished **(G)** animals stained with hematoxylin and eosin. Bars = 50 μm. **(D, F, H):** Sections of prostate tissue from intact **(D)**, castrated **(F)**, and castrated and androgen-replenished **(H)** animals immunohistochemically stained using an antibody against α-smooth muscle actin. Sections were counterstained with hematoxylin. Bars = 50 μm. Abbreviations: w, weeks; RC, renal capsule; UGM, urogenital sinus mesenchyme.

To determine the sensitivity to androgens, donor cells isolated from different regions of ducts of androgen-replete animals were implanted under the RC of (a) androgen-replete recipients and harvested after 8 weeks, (b) castrated recipients

and harvested after 16 weeks, and (c) castrated recipients and harvested after 8 weeks of androgen deprivation followed by 8 weeks of androgen supplementation (Fig. 4A). Each group received donor cells isolated from one of the following: all regions of ducts, the proximal, intermediate, or distal region, or the urethra (Fig. 1A). Very little growth was noted when cells from any region were implanted in castrated recipients (Fig. 4B, center bar in each group). Cells isolated from both the proximal region and the urethra maintained full regenerative capacity through 8 weeks of androgen deprivation; the amount of prostatic tissue that was formed after subsequent exposure to androgens was comparable with that noted when cells from the proximal region or the urethra were implanted in androgen-replete animals (Fig. 4B, supplemental online Fig. 5). In contrast, the ability of cells isolated from the intermediate and distal regions to regenerate prostatic tissue was severely compromised by androgen deprivation (Fig. 4B, supplemental online Fig. 5), indicating that these regions contained cells that require androgen for survival. Intermediate or distal cells formed more tissue in intact animals (65 ± 26 mg or 25 ± 19 mg, respectively) compared with animals maintained in an androgen-deficient state for 8 weeks and subsequently exposed to androgens for an additional 8 weeks (5 ± 3 mg or 3 ± 1 mg respectively; $p <$ .001; Fig. 4B, supplemental online Fig. 5). Cells isolated from all regions of the prostate (Fig. 1A) could regenerate more prostatic tissue (Fig. 4B, supplemental online Fig. 5) than could intermediate or distal cells. This is likely due to the proximal cells included in this fraction. These data indicate that cells in the proximal region can survive prolonged periods of androgen deprivation and retain full regenerative potential and that urethral cells have similar properties.

Histological examination of the sub-RC tissue removed from intact animals showed prostatic ducts containing basal and luminal cells (Figs. 1E, 1F, 4C). The ducts were enveloped by a thin band of smooth muscle (Fig. 4D) as is noted in normal prostate [23]. When implants of either intermediate or distal cells were placed in androgen-deprived animals, no evidence of epithelial cells or ducts was noted (data not shown). However, when implants originating from proximal or urethral cells were examined, small rudimentary ducts were noted 16 weeks after androgen deprivation (Fig. 4E, 4F). This indicates that some epithelial cells survived and formed small ductal structures in the absence of androgen. These ducts are the likely source of the primitive cells from which the tissue regenerated after androgen administration. Histological examination of the tissue arising from proximal or urethral cells after androgen deprivation and subsequent regeneration indicated an extensive ductal network surrounded by significantly more smooth muscle tissue (Fig. 4H) than was noted in intact animals (Fig. 4D). These data show that two populations of cells capable of regenerating prostatic tissue in androgen-replete animals are present in the prostate. The first population resides in the proximal region and the urethra, and the survival of these cells does not require the presence of androgens. The second population (with more limited growth potential) is found in the remaining ductal regions and requires androgen for survival.

Castration results in the involution of the gland and the loss of significant numbers of cells primarily from distal regions, with the proximal region being least affected [1, 2]. We therefore compared the phenotype and growth potential of cells

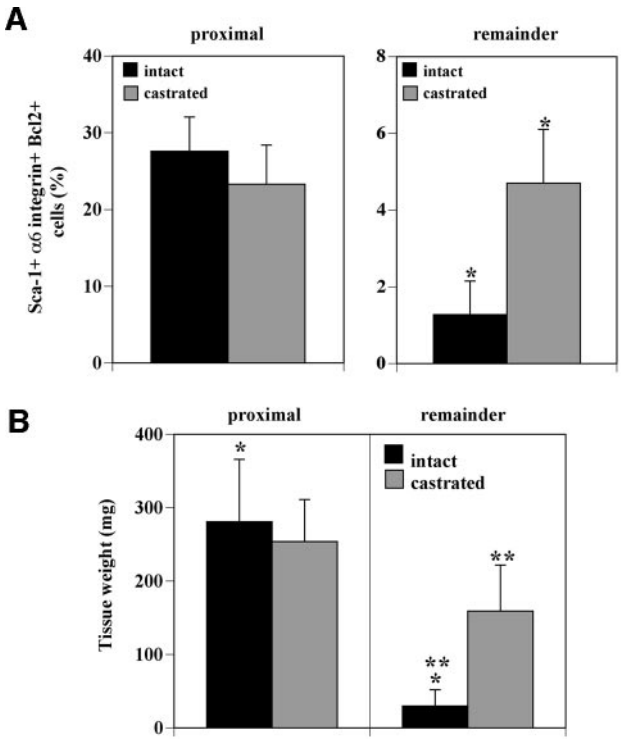

Figure 5. Castration enriches the primitive cells in the remaining regions of ducts. **(A):** Three-color FACS analysis was performed to determine the percentage of Sca-1$^+$α6 integrin$^+$Bcl-2$^+$ cells in the proximal ($n = 3$) and remaining ($n = 3$) regions of ducts in intact and castrated animals. ∗, $p < .04$. Each bar represents the mean ± SD. **(B):** Cells (10^5) from the proximal ($n = 6$) or remaining ($n = 7$) regions of ducts from intact or castrated animals were combined with UGM cells (2.5×10^5) and implanted under the RC of intact animals. Grafts were harvested after 8 weeks. ∗, $p < .002$; ∗∗, $p < .001$. Abbreviations: FACS, fluorescence-activated cell sorting; RC, renal capsule; UGM, urogenital sinus mesenchyme.

isolated from the proximal and remaining regions of ducts from androgen-replete and castrated animals to determine whether cells with primitive regenerative features were enriched in the remaining regions of ducts after involution.

We have previously shown that cells coexpressing the antigens Sca-1, α6 integrin, and Bcl-2 are concentrated in the proximal region of ducts [5]. We therefore determined the coexpression of these antigens on cells in different ductal regions in androgen-replete and castrated animals to ascertain whether their incidence was altered by involution of the gland (Fig. 5A). Castration resulted in a 3.4-fold increase ($p < .04$) in cells that coexpressed these antigens in the remaining regions of ducts, whereas similar numbers of cells in the proximal region of intact and castrated animals coexpressed these antigens.

We next compared the proliferative potential of cells isolated from different regions of ducts from both androgen-replete and castrated animals. Cells isolated from the remaining regions of ducts of castrated animals formed 5.3-fold more sub-RC tissue than did cells isolated from the remaining regions of ducts of intact animals ($p < .001$), whereas cells isolated from the proximal region from intact and castrated animals formed similar amounts of tissue (Fig. 5B). This indicates that involution of the gland is accompanied by the enrichment of those cells with

proliferative potential in the remaining regions of ducts, whereas castration does not affect the proportion of cells capable of forming prostatic tissue in the proximal region. These data show that castration enriches for cells with a primitive phenotype in the remaining region of ducts, indicating that the more mature cells die during involution. The proportion of primitive cells in the proximal region is unaffected by androgen levels, indicating that cells in this region survive androgen ablation.

Cells from the Proximal Region Can Be Serially Passaged In Vivo

Passage of cells isolated from undissected recombinant tissue (i.e., all regions). Because stem cells have high regenerative potential, we determined whether the RC tissue obtained after implantation of proximal cells could be serially passaged in vivo more frequently than tissue arising from distal cells. Cells (10^5) were isolated from the proximal and distal regions of primary prostates and implanted under the RC (Fig. 2A). Prostatic tissue obtained from proximal and distal cell sub-RC implants was weighed and digested. Cells (10^5) from each of these digests were combined with UGM and re-implanted under the RC of a second animal (P2). This process was repeated until no prostatic tissue growth was noted (Fig. 2A, 2B). Cells from the proximal region can be serially passaged four times, whereas distal cells can be passaged twice. In addition, as noted previously in primary implants (Fig. 1), cells isolated from the proximal region formed larger amounts of prostatic tissue at each consecutive passage than did cells isolated from the distal region, indicating that proximal cells were capable of more extensive division than distal cells.

Passage of cells isolated from the* proximal *region of* recombinant *tissue. We wished to determine whether the sub-RC prostatic tissue formed an "organ" that maintained a physiologically distinct proximal-distal ductal axis similar to that observed in the prostate and whether cells isolated from different regions of the sub-RC graft displayed similar disparate growth properties to that noted in primary prostatic ducts. Cells were therefore isolated from proximal and distal ductal regions of the primary prostate (Fig. 3A, 3B) and implanted under the RC. Microdissection of the sub-RC tissue mass obtained from proximal cells (Fig. 3A, 3C) revealed an interconnected series of ducts consisting of proximal and distal regions very similar to that obtained from the primary prostate (Fig. 3B). Quite remarkably, when these ducts were dissected into proximal and distal regions and isolated cells were reimplanted under the RC, the cells from the proximal region once again formed large amounts of prostatic tissue (228 ± 83 mg) compared with cells obtained from the distal region (5 ± 4 mg; $p < .02$; Fig. 3A, 3D, P2). The tissue obtained from P2 proximal cells also had a visible proximal-distal ductal axis and was similarly dissected and reimplanted. Proximal cells again formed more (76 ± 23 mg) prostatic tissue than did distal cells (49 ± 19 mg; P3, $p < .04$). The tissue obtained from P3 proximal cells was again dissected and reimplanted. A small amount of sub-RC prostatic tissue growth was noted in two (9.3 ± 1.6 mg) out of six proximal cell implants (P4).

These data indicate that primitive proximal cells are programmed to re-establish a proximal-distal ductal axis under the RC (Fig. 3C) and have extensive regenerative capacity, as they can be passaged through four generations of sub-RC grafts.

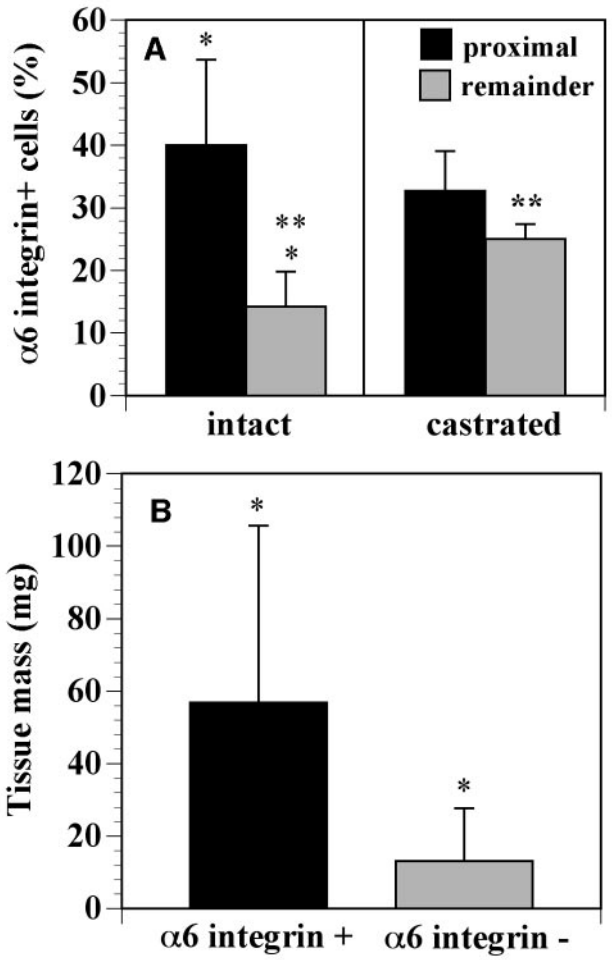

Figure 6. α6 Integrin-expressing cells are enriched in the proximal region of ducts and form more prostatic tissue under the RC than those depleted of this antigen. **(A):** The expression of α6 integrin in the proximal and remaining regions of intact ($n = 6$) and castrated ($n = 4$) animals. *, $p < .002$; **, $p < .02$. **(B):** Proximal α6 integrin-enriched ($n = 11$) and α6 integrin-depleted ($n = 15$) cells (10^5) were combined with UGM cells (2.5×10^5) and implanted under the RC. Grafts were harvested after 8 weeks. *, $p < .002$. Each bar represents the mean ± SD. Abbreviations: RC, renal capsule; UGM, urogenital sinus mesenchyme.

Cells isolated from the proximal region of successive grafts form considerably more sub-RC tissue (Fig. 3; 228 mg P2, 76 mg P3) than those isolated from similar passages from the entire graft (Fig. 2; all regions, 78 mg P2, 2 mg P3). This confirms that the proximal region of sub-RC tissue is similar to the proximal region of a primary prostate gland because it is also enriched in primitive cells with high regenerative capacity.

α6 Integrin-Expressing Proximal Cells Have High In Vivo Proliferative Potential

α6 integrin is present on primitive cells from a number of origins [24–29], including the prostate [5], and the gene for this antigen was the only common gene identified in a study using transcriptional profiling to identify genes expressed by stem cells of embryonic, neural, hematopoietic, and retinal origin [30]. To determine whether cells expressing this antigen were enriched after castration and to ascertain whether α6 integrin-

expressing cells had greater regenerative capacity than cells not expressing this antigen, we performed two sets of experiments. We first determined expression of this antigen on proximal and remaining cells from androgen-replete and castrated animals. The proximal region of intact animals contained 2.8-fold ($p < .002$) more α6 integrin-expressing cells than the remaining regions of ducts (Fig. 6A). Castration resulted in an increase in α6 integrin-expressing cells in the remaining ductal regions (from 14.2% ± 5.6% to 25.1% ± 2.4%; $p < .02$) without affecting the incidence of these cells in the proximal region, indicating that involution of the gland was accompanied by an enrichment in α6 integrin-expressing cells. We next isolated cells expressing α6 integrin from the proximal region and determined their regenerative potential in vivo. Cells enriched for α6 integrin expression formed 4.3-fold more prostatic tissue (57 ± 48 mg) than did cells depleted of this antigen (13 ± 14 mg; $p < .002$, Fig. 6B), indicating that α6 integrin-expressing cells have greater regenerative potential than those cells lacking this antigen.

DISCUSSION

We show that ductal regions of androgen-replete animals vary markedly in their ability to survive androgen ablation. Two distinct populations of cells with different androgen sensitivities are capable of sub-RC organ reconstitution. The proximal region and the urethra contain cells that regenerate prostatic tissue far more robustly than cells isolated from the intermediate or distal regions of ducts. These cells survive androgen ablation and regenerate prostatic tissue fully once androgens are re-administered. Both these attributes would be expected in a stem cell population. A second population, residing in the intermediate and distal regions of ducts, is capable of a more limited organ reconstitution and is androgen-sensitive in terms of their survival. Cells in these regions are unable to regenerate prostatic tissue after androgen deprivation. These may be the cells that are referred to as transit-amplifying cells and that are considered to be a post-stem cell population. Because all of the regions of the ducts express equivalent levels of androgen receptors and 5-α-reductase [31], the differences in sensitivity to androgens are not due to regional variations in expression of these molecules.

Although we and others [32] show that the sizes of the grafts are dependent on the numbers of epithelial cells used in the assay, some [15] have reported that the amount of acinar growth was dependent on the mesenchymal tissue and not the epithelial component. We are uncertain of the explanation for these differences. A possible explanation could be that we used digests of prostate cells obtained from adult animals whereas Chung and Cunha [15] used fragments of epithelium of embryonic origin.

We also show that, in the remaining regions of ducts, castration results in the enrichment of primitive cells that are capable of forming prostatic tissue. Cells removed from the remaining regions of ducts of an involuted prostate formed more tissue under the RC than did cells isolated from these regions of an androgen-replete prostate. Castration results in the loss of large numbers of epithelial cells from the distal regions of ducts [1, 2]. We show that this is accompanied by an increase in the proportion of cells in these regions that coexpress three antigens (Sca-1, α6 integrin, and Bcl-2) shown to be expressed by primitive prostate cells [5]. The remaining regions of ducts of intact animals have very few of these primitive cells. These results support the idea that the cells lost as a result of castration are differentiated nonregenerative cells. It is interesting that isolated cells removed from remaining regions of ducts and placed in castrated recipients for 2 months followed by androgen administration could not reconstitute prostatic tissue under the RC whereas cells isolated from the remaining ductal regions of an involuted prostate removed from an androgen-deprived animal can regenerate prostatic tissue. This discrepancy may be due to the enrichment of primitive cells in the remaining ductal regions that follows castration and involution as this process results in the loss of differentiated cells. Thus, a larger number of primitive cells would be present in the remaining regions of castrated prostates than in the remaining regions of androgen-replete prostates.

We also show that primitive cells in the proximal region are programmed to regenerate a proximal-distal ductal axis through consecutive passages in a prostate reconstitution assay. The most definitive test of stem cell function is their ability to reconstitute an organ. Serially transplanted bone marrow can reconstitute lethally irradiated mice [33, 34], and the number of successful serial transfers is dependent on the size of the grafts and the time intervals between transfers [35].

The appearance of the dissected sub-RC tissue was remarkably similar to that noted in an intact prostate gland (Fig. 3B, 3C), and primitive cells were concentrated in the proximal regions of both the intact gland and the sub-RC tissue. This indicates that primitive prostate cells are programmed to regenerate an organ with regional differences in regenerative capacity. Isolated cells from the proximal regions of sequential grafts could be passaged four times before senescence and formed large amounts of prostatic tissue (228 ± 83 mg) that greatly exceeded the size of a primary prostate gland (11 ± 1 mg). This indicates that primitive cells in the proximal region of the sub-RC grafts have considerable regenerative capacity. It is not surprising that primitive proximal cells cannot be passaged indefinitely; experimental evidence indicates that hematopoietic stem cells show signs of aging and have a limited functional lifespan [36]. This is likely to be an important discriminator between healthy stem and tumor stem cells.

α6 Integrin is the only common protein expressed by stem cells of a number of origins and is present on the primitive cells of different tissues [5, 24–30]. We show that in the prostate the expression of α6 integrin alone is also indicative of a primitive phenotype as the proportion of cells expressing this antigen in the remaining regions of ducts is increased after castration. In addition, proximal cells enriched for α6 integrin expression have 4.3-fold greater proliferative potential than those depleted of this antigen, indicating that α6 integrin expression identifies cells with enhanced regenerative potential in vivo as has been recently shown for cells expressing Sca-1 [5, 6]. Putative human prostate stem cells have also been shown to express high levels of another member of the integrin family, namely α2 integrin [37].

Stem cells generally reside in specialized niches that form a microenvironment that maintains their primitive phenotype. The proximal region of ducts provides a protective niche for prostatic stem cells which may permit them to survive in the absence of androgen. This region is least affected by castration in terms of apoptosis and cell loss [1, 2], as indicated by the similar incidence of cells in this region that coexpress Sca-1, α6 inte-

grin, and Bcl-2 in androgen-replete and castrated animals. The proximal region also has the highest levels of telomerase [38], which is associated with germinative compartments of many self-renewing tissues [39, 40]. The cells in the proximal stem cell niche are kept quiescent by high levels of TGF-β signaling in this region (compared with low levels of signaling in distal regions), and they are protected from the TGF-β-mediated apoptosis that follows androgen withdrawal by high levels of Bcl-2 expressed by cells in the proximal region [3].

SUMMARY

The data in this manuscript show that two distinct cell populations with different androgen sensitivities are located in different regions of prostatic ducts. The proximal region contains primitive cells with attributes of stem cells. They survive in the absence of androgen and are able to regenerate large amounts of prostatic tissue after androgen replenishment. The remaining regions of ducts contain cells with limited regenerative capacity that are unable to withstand androgen withdrawal. Interestingly, primitive proximal cells are programmed to maintain a functional proximal-distal ductal axis through successive passages in a sub-RC prostate reconstitution assay. The prostate is an androgen-sensitive organ that is the site of considerable pathology as both prostate cancer and benign prostatic hyperplasia are common diseases. Because prostate carcinoma evolves into an androgen-independent disease that may reflect the emergence of cells with stem-like properties, the identification of cells within regions of prostatic ducts capable of withstanding androgen ablation may lead to advances in elucidating the biology of proliferative prostatic diseases. Because the prostate is a nonessential organ, it may be possible to develop a therapy that targets and ablates the stem cell compartment prior to the development of proliferative abnormalities in this gland.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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